GENERATION OF NATURAL KILLER CELLS FROM UMBILICAL CORD BLOOD STEM CELLS, CHARACTERISATION AND APPLICATION FOR IMMUNOTHERAPY

Martha Elia Luevano Salinas

A thesis submitted to the University College London for the degree of Doctor of Philosophy

2013

Anthony Nolan Research Institute
Cancer Institute
University College London (UCL)

Declaration

I, Martha Elia Luevano Salinas, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:……

Date:……..

This thesis is dedicated to my family and especially to my beloved life companion and husband Brian Davis.

Abstract

Adoptive (Natural killer) NK cell therapy relies on the use of a large amount of NK cells that are cytotoxic, and yet not exhausted. For this purpose, NK cells can be isolated from cord blood, peripheral blood or generated *in vitro* from haematopoietic stem cells (HSC). *In vitro* generation of high numbers of activated NK cells using HSC would facilitate multiple infusions and treatment of patients with large tumour burden, allowing to by-pass the limitations of NK cell numbers and activation state of blood-derived NK cells. However, comprehensive studies about the use of fresh or cryopreserved HSC and of different HSC sources for protocols of NK cell production *in vitro* have yet to be performed. The aim of this thesis was to investigate these variables and establish an optimal protocol for the generation of NK cells *in vitro*.

This work investigated the use of a published protocol and a modified version using only IL-15 for the last weeks of culture for NK cell generation; moreover, the comparison of NK cells derived from fresh cord blood stem cells (CBSC) and frozen CBSC and a different HSC source, mobilised peripheral blood stem cells (PBSC), was performed. Using this protocol, we showed that frozen CBSC generated higher NK cell numbers expressing activating receptors, lacking killer-cell immunoglobulin like receptor expression but with better immunoregulatory and cytotoxic properties compared to NK cells from fresh CBSC and PBSC cultures. More than half of the NK cells generated *in vitro* from all HSC types expressed the myeloid-marker CD33; blocking of this marker did not impact on NK cell functions. Finally, CBSC and PBSC showed a different threshold for NK cell activation with interleukin 12. In conclusion, our study provides evidence that frozen CBSC are a suitable source of HSC for NK cell generation *in vitro* compared to fresh CBSC and frozen PBSC.

Acknowledgements

I would like to thank first of all Professor Alejandro Madrigal for giving me the opportunity to join the lab and start one of the most amazing journeys I have ever had, for his precious professional advices and the cultural and artistic insights. This project would have not been able to be completed without the financial support from CONACyT, SEP and Anthony Nolan.

I want to express how thankful I am for Aurore's supervision, her example as a creative and talented researcher worth imitating. I appreciate the thoughtful guidance in every aspect of this thesis and the support in each step of my PhD, from the beginning until now.

I would like to thank Professor Steve Marsh for all the fun provided during lunchtime for the past three years, the good advices and great stories shared. I am sure we will have them from time to time in the future and by then I would have forgotten the British pronunciation of each word you insisted on learning.

The samples used during this thesis were provided by two cord blood banks, Nottingham and Barcelona. I would like to thank the teams that ensured that all samples were provided as requested, and particularly Dr. Susana Garcia and Dr. Sergio Querol. Additionally, I would like to thank Dr. Kwee Yong for providing the mobilised peripheral blood stem cell samples and especially to Nadia for her friendship and for making this collaboration possible, to Dr. Mike Blundell and Prof. Adrian Thrasher for their collaboration in the animal model experiments.

I sincerely thank Steve Cox for taking the time to read this thesis and for the precious advice. This project would have not been the same without the current and past students and staff in the Nolan. I have experienced a true teamwork with each one of the students and staff from the lab. We have shared reagents, bench space, samples and work. I cannot thank enough the key people that greatly helped during the NK cell culture experiments: Anna Bobana, Isabela and Sophie. I also would like to give special thanks to Michelle and Rehab for continuously sharing of cord blood samples for NK cell isolation. Big thanks for all the laughs, dancing and singing provided by Fuiyee, Isabela and Rehab. Special thanks to Michelle for introducing me to the facilitation world and for the great personal and academic advice and perfect teamwork

during the PhD. To my “regia” friend Isabela for sharing amazing years of laughs, good food, advice and great experiences!

All the late night shifts would have never been the same without the great company of Cesar, Esteban, Isabela, Sameer, Sofia and Christian. The constant supply of cookies, great evening talks, laughs, stories or even squash matches were truly key for the successful completion of the experiments.

Thanks to all the volunteers that donated blood (willingly or not). To Bronwen, Neema, Sameer, Angus, Nush, Rob, Jason and James for sharing this amazing experience. Special thanks to Jennifer for all the training tips and the morning runs in Hampstead Heath, and of course for the HLA typing of my samples. To Hazel and Mark for making sure every single reagent was always available and on time, for all the advice and great talks. To Pauline for her precious help during all my PhD, paperwork, signatures, document scanning and for great talks and moments.

I am thankful to my friends that are not part of UCL or Anthony Nolan; Berenice, Zulema, Diana, Pete, Vanesa, and especially to my best training partner and friend Silvia Bolanos, To my family in Mexico, for their continuous support and love, for all the luggages sent full of fried beans, corn tortillas and “machacado”. Thanks to my sister and best friend Elvaluisa for all the times shared during the PhD, for being a great listener and provider of smiles. To my mom and dad, who have been the greatest examples to follow, for your motivation, love and support during all these years, thank you for never giving up on me. And finally, to the very special person that came in the middle of my PhD as the best gift of God, Brian Davis. I cannot thank you enough for all your patience and comprehension, for your companion during good and bad times, for your continuous support and encouragement and for the love you give me.

“Delight yourself in the LORD, and he will give you the desires of your heart”

Psalms 37:4

Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

List of Tables

Chapter 1

Chapter 2

Abbreviations

7AAD	7-Amino-actinomycin D
ADCC	Antibody-dependent cell-mediated cytotoxicity
AICL	Activation-induced C-type lectin
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
APC	Antigen presenting cells
BAT-3	HLA-B-associated transcript 3
BM	Bone Marrow
BMT	Bone marrow transplant
BSA	Bovine serum albumin
CB	Umbilical cord blood
CBMCs	Cord blood mononuclear cells
CBSC	Cord blood stem cells
CBT	Cord blood transplantation
CEA	Carcinoembryonic antigen
Cebp-g	CCAAT-enhancer binding protein
CIBMTR	Centre for International Blood and Marrow Transplant Research
CLIP	Class-II associated Ii peptide
CLPs	Common lymphoid precursors
CML	Chronic Myeloid Leukaemia
CMPs	Common myeloid precursors
CR	Complete remission
CTLs	Cytotoxic T lymphocytes
DAP12	DNAX-activating protein 12 kDa
DC	Dendritic cell
DL1	Delta-like 1
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsuphoxide
dNK	Decidua NK
DR	Death receptor
E:T	Effector-to-target
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency

eNK	Endometrial NK
ER	Endoplasmic reticulum
FADD	Fas-Associated Death Domain
FBS	Foetal bovine serum
FSC	Forward scatter
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte–macrophage colony-stimulating factor
GMP	Good manufacturing practices
GPCRs	G protein-coupled receptors
GvHD	Graft versus host disease
GvL	Graft versus leukaemia
h	Hours
HCMV	Human cytomegalovirus
hESC	Human embryonic stem cells
Hlx	H2.0-like homeobox
HPC	Haematopoietic progenitor cell
HSCs	Haematopoietic stem cells
HSCT	Haematopoietic stem cell transplantation
HSP	Heparan sulfate proteoglycans
HSV	Herpex simplex virus
iCD3ε	Intracellular CD3ε
Ii	Invariant chain
IL	Interleukin
ILTs	Ig-like transcripts
iNK	Immature NK
Iono	Ionomycin
iPSC	Induced pluripotent stem cells
Irf	Interferon regulatory factor
IS	Immune synapse
ISHAGE	International Society of Hematotherapy and Graft Engineering
ITAM	Immunoreceptor tyrosine-based activation motifs
ITIM	immunoreceptor tyrosine-based inhibition motif
KIRs	Killer-cell immunoglobulin-like receptors
KLRG1	Killer cell lectin-like receptor G1
LAK	lymphokine-activate killer
LAMP	Lysosome-associated membrane protein
LAT	Linker for activation of T cells
LNs	Lymph nodes

LTi	Lymphoid tissue inducer
M6P	Mannose-6 phosphate
MCMV	Murine cytomegalovirus
Mef	Myeloid ELF1-like
MFI	mean fluorescent intensity
MHC	Major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
miRNAs	micro-RNAs
Mitf	Microphthalmia transcription factor
µl	Microliters
ml	Millilitres
mNK	Mature NK
MRCC	Metastatic renal cell carcinoma
MTOC	Microtubule organising centre
NCRs	Natural cytotoxicity receptors
NHL	Non-Hodgkin lymphoma
NK	Natural Killer
NK-ireg	NK immunoregulatory
NKP	Natural killer cell precursor
NKR-PA1	NK cell receptor protein 1A
NOD-SCID	Non-obese diabetic severe combined immune deficient
NSCLC	Non-small cell lung cancer
NTAL	Non-T-cell activation linker
PAMPs	Pathogen-associated molecular patterns
PB	Peripheral blood
PBMCs	Peripheral blood mononuclear cells
PBSC	Peripheral blood stem cells
PCL- γ	Phospholipase C
PI3K	Phosphatidylinositol-3 kinase
PIP_3	Phosphatidyl-inositol-3,4,5-trisphosphate
PMA	Phorbol 12-myristate 13-acetate
PPRs	Pattern recognition receptors
PTP	Protein tyrosine phosphatases
Pyk2	Proline-rich tyrosine kinase 2
qPCR	Real time PCR
ROS	Reactive oxygen species
SCF	Stem cell factor

SD	Standard deviation
SDF	Stromal-cell derived factor
SIGLECs	Salic-acid-binding immunoglobulin-like lectins
siglecs	Sialic acid-binding Ig-like lectins
SLTs	Secondary lymphoid tissues
SSC	Side scatter
TAP	Transporter associated with antigen presentation
T_{CM}	Central memory T cells
TCR	T cell receptor
T_{EM}	Effector memory T cells
TFs	Transcription factors
T_H	Helper T lymphocytes
TLRs	Toll-like receptors
TRAIL	TNF-related apoptosis-inducing ligand
Tregs	Regulatory T cells
uNK	Uterine NK
VEGF	Vascular endothelial growth factor
VZV	Varicela Zoster Virus

Chapter 1: Introduction

1.1 The immune system

The immune system is a network of cells, cell products, tissues and organs that protect the body against pathogens, non-self and transformed cells. The immune system is complex and comprises numerous physical defences and cells with a specific function. Traditionally, the immune system is divided into two categories, the innate immune system (non-specific) and the adaptive immune system characterised by specificity and memory. The following is a brief description of these two arms of immunity.

1.1.1 Innate immunity

The innate immune system refers to physical barriers such as the skin and mucosa as well as non-specific cellular defences triggered immediately or within hours after exposure to any foreign microbe/antigen/substance. The main components of the innate immunity include physical barriers, effector proteins, effector cells and cytokines (figure 1.1). The first line of defence is provided by the skin and mucosae, which act as physical barriers and anatomical traps preventing the entrance or growth of most microbes. They can contain antibacterial enzymes such as defensins and cathelicidins that destroy bacterial membrane walls and physically trap microbes (figure 1.1). The second line of defence involves antimicrobial proteins and phagocytic cells able to recognise broad classes of pathogenic organisms through germ-line encoded antigen-specific pattern recognition receptors (PPRs) such as Toll-like receptors (TLRs). Another class of innate immune lymphocytes are natural killer (NK) cells, which are the focus of this thesis; a detailed description of this cell subset is provided in the following sections. A variety of proteins are also involved in the defence against pathogens such as cytokines.

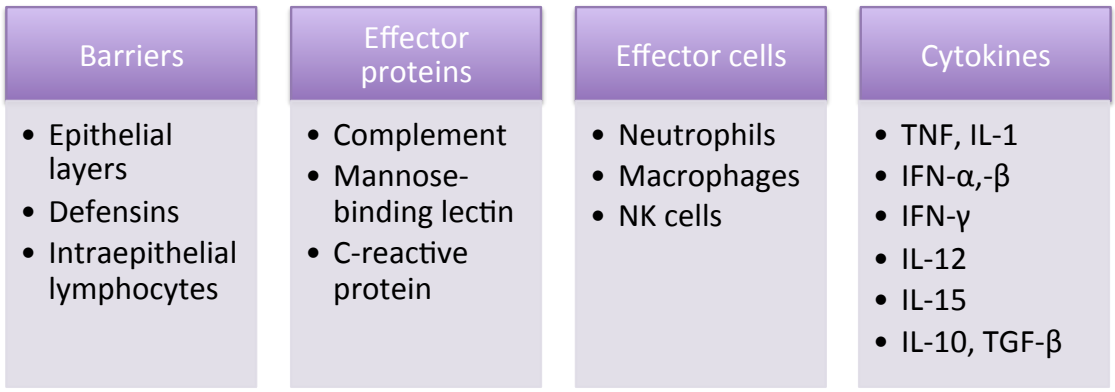


Figure 1.1. Components of the innate immune system.

While often effective, these non-specific responses are not impregnable, if they fail to eliminate the foreign invader, a highly specific immune response needs to be mounted by the adaptive immunity.

1.1.2 Adaptive immunity

The fundamental features of the adaptive immune system are: specificity, diversity, memory, clonal expansion, specialisation, homeostasis and non-reactivity to self (Abul K. Abbas, 2011). There are two types of adaptive immune responses: humoral and cell-mediated immunity. The humoral immunity is mediated by antibodies produced by B lymphocytes. The cellular-mediated immunity (cellular immunity) is mediated by T lymphocytes. Intracellular invaders, such as viruses or bacteria, are able to survive and proliferate inside the host cells, where antibodies have no access. In this case, cellular immunity supports the destruction of these infected cells. Figure 1.2 shows the main components of the cellular and humoral immunity and how they interact, additionally a summary of both immune responses is shown in the following sections.

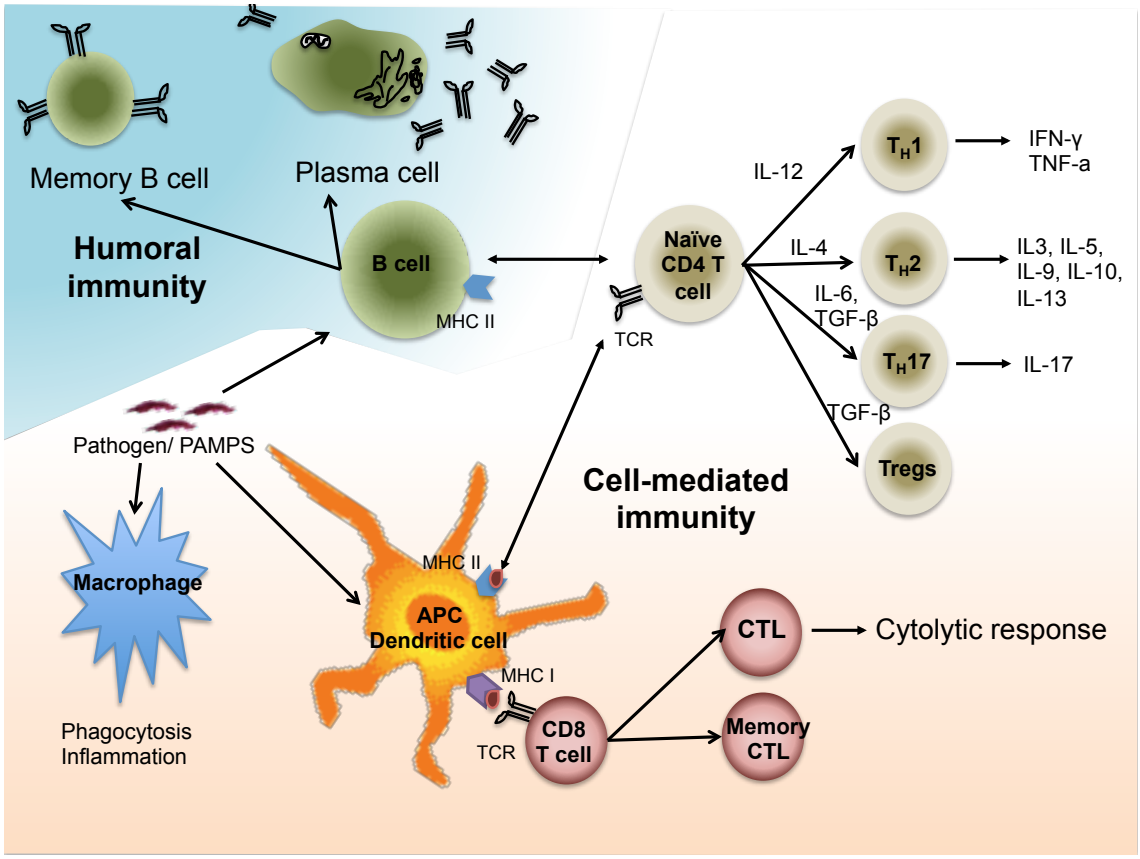


Figure 1.2. Immune system interactions. The immune system consists of several cellular interactions that lead to different functional responses by different immune cells. Numerous cell surface receptor–ligand interactions, cytokines, and inflammatory molecules coordinate and affect the immune response. Antigen presenting cells such as dendritic cells can interact with CD8 T cells to stimulate cellular (cytolytic) immune responses, or with CD4 T cells to prime humoral (antibody) based responses. A CD4 T cell can differentiate into a variety of effector subsets, which include those that stimulate B cells to produce antibodies or those that help activate other immune clearance and regulatory processes. Modified with permission from Elsevier. (Look *et al.*, 2010)

Antigens

The term antigen was previously used for any molecule that induced the generation of a specific antibody by B cells. However, now antigen is defined as any substance that can induce an immune response. Antigens can be a natural substance or man-made (John Hall, 2010).

Cell-mediated immunity: T cells

T cells are lymphocytes that mediate cellular immunity. The two major T lymphocyte subsets are helper T lymphocytes (T_H) and cytotoxic T lymphocytes (CTLs), which express the antigen receptor αβ. Most T_H cells express CD4 and most CTLs express CD8 (Abul K. Abbas, 2011). T cells express TCR (T cell receptor); a molecule restricted to the recognition of peptides bound to major histocompatibility complex

(MHC) molecules on antigen presenting cells (APCs). Although other cells can act as APCs (B cells and macrophages), dendritic cells (DCs) are the most effective at presenting antigens. CD8 T cells recognise peptides presented by class I MHC molecules while CD4 T cells recognise peptides via class II MHC molecules. The TCR engagement triggers positive or negative signals that result in cellular proliferation, cytokine production or even cell death (Lin and Weiss, 2001). DCs can activate naïve T cells, which are able to differentiate into different cell classes that produce specific cytokines and have special effector functions. The activation of naïve CD4 T cells can lead to the differentiation into T_H1, T_H2, T_H17 and Tregs (Mosmann *et al.*, 1986; Korn *et al.*, 2009) (figure 1.2). T_H1 cells protect against intracellular pathogens and T_H2, against extracellular pathogens. T_H17 are characterised by the production of IL-17, a proinflammatory cytokine, and Tregs are immunosuppressive and maintain self-tolerance (Sakaguchi, 2004). After the expansion phase most T cells will die, however, a memory cell pool is maintained for a faster and stronger secondary immune response. Protective memory is mediated by effector memory T cells (T_{EM}) and central memory T cells (T_{CM}). T_{EM} cells have immediate effector functions and T_{CM} have higher sensitivity to antigenic stimulation compared to naïve T cells (Lanzavecchia and Sallusto, 2000). Similarly, activation of naïve CD8 T cells allows its differentiation into activated CD8 T cells that acquire cytotoxic properties or differentiate into memory CD8 T cells (resident memory $CD8^+$ T cells and effector-like memory $CD8^+$ T cells) (Hamilton and Jameson, 2012).

Humoral immunity: B cells

B cells have a unique function in adaptive immunity, as they are the only cells able to synthesise and secrete antibodies. Antibodies are glycoproteins that mediate humoral immunity through binding to a wide range of antigen structures with high affinity and strength. Antibodies can mediate effector functions, including neutralisation of microbes, activation of the complement system, opsonisation and antibody-dependent cell-mediated cytotoxicity (ADCC). In mammals, five antibody classes (IgA, IgD, IgE, IgG, IgM) have been described. Each one has a different function and distribution within the body. A naïve B cell can circulate throughout the body and carries out immune surveillance via its surface-bound IgM or IgG. The B cell response to antigens usually requires interaction with T_H cells. B cells can act as APCs to T cells, TCRs on T_H cells bind to the MHC-II-antigen complex on B cells resulting in T cell activation and enabling the release of a second activating signal to the B cell. B cell activation occurs when a naïve B cells encounters its antigen and leads to proliferation/expansion of the antigen-specific cells and further differentiation generating effector plasma B cells that

secrete specific antibodies (figure 1.2). Additional signals regulate the plasma cell antibody switching and regulate antibody production. After the initial activation, some B cells develop into memory B cells that express high affinity immunoglobulins (usually IgG) and survive for long periods of time enabling a quicker second immune response (Abul K. Abbas, 2011).

1.2 Major histocompatibility complex

MHC class I and class II are similar in function; they present peptides to $CD8^+$ and $CD4^+$ cells, respectively. These peptides have a different origin, intracellular for MHC class I and exogenous for MHC class II (Rock *et al.*, 2004; Watts, 2004).

MHC class I molecules are encoded by the heavy chain human leukocyte antigen (HLA)-A, HLA-B or HLA-C and the β2-microglobulin invariant subunit (β2m) and are present on all nucleated cells. Antigens are degraded in the cytosol by proteasomes and the transporter associated with antigen presentation (TAP) translocates the resulting peptides into the endoplasmic reticulum (ER). Within the ER, the MHC class I heterodimer is formed with the assistance of chaperones and tapasin, a glycoprotein that mediates interaction between TAP and MHC class I molecules. This complex is then translocated from the ER to the cell surface (Vyas *et al.*, 2008).

Similar to MHC class I, MHC class II molecules are encoded by three polymorphic genes (HLA-DR, HLA-DQ and HLA-DP) able to bind different peptides. MHC class II molecules are mainly expressed by APCs, such as dendritic cells (DCs), B cells and macrophages. MHC class II chains are assembled in the ER and associated with the invariant chain (Ii). The complex Ii-MHC class II is transported to a late endosomal compartment where it is digested leaving a residual class-II associated Ii peptide (CLIP) (Busch *et al.*, 2005). This complex is then transported to the cell membrane to present their cargo to $CD4^+$ T cells.

1.3 Natural Killer cells

Almost forty years ago, Kiessling (Kiessling *et al.*, 1975a; Kiessling *et al.*, 1975b) and Herberman (Herberman *et al.*, 1975a; Herberman *et al.*, 1975b) observed a lymphocyte subset able to lyse target cells in the absence of a priori stimulation. Consequently, these cells were named “natural killer” (NK) cells and are large non-phagocytic granular lymphocytes of the innate immune system present in both,

lymphoid organs and non-lymphoid organs (Trinchieri, 1989; Cooper *et al.*, 2004). Initially, NK cells were regarded as “experimental artefacts” in T cell cytotoxicity assays, however, later it was appreciated that NK cells displayed different morphology depending on their activation status (Dvorak *et al.*, 1983), rendering them difficult to detect according to size and morphology. NK cells are now characterised by their surface expression of CD56 and CD16 and absence of CD3 (Ritz *et al.*, 1988; Lanier *et al.*, 1989) and comprise around 15% of all lymphocytes.

NK cells are part of the innate immune system and mediate the response against viruses, parasites, bacteria and tumour cells (Moretta *et al.*, 2002; Raulet, 2004). Additionally, NK cells contribute to the adaptive immune response by linking innate and the adaptive immunity through their receptor FcγRIIIA (CD16). These responses are mediated through two main effector functions, direct lysis of target cells and the secretion of cytokines. The mechanism by which NK cells discriminate between healthy and target cells was discovered in 1986 by Karre *et al*. During this work, the observation that NK cell cytotoxicity inversely correlates with the level of MHC class I expression on target cells led to the "missing self hypothesis" (Karre *et al.*, 1986; Karre, 2002).

Natural Killer cell subsets

Two distinct subsets of human NK cells have been identified based on CD56 expression: $CD56^{dim}$ and $CD56^{bright}$ NK cells (Lanier *et al.*, 1986). The majority of human peripheral blood (PB) NK cells are $CD56^{dim}$ (90%) and expresses high levels of CD16; a minority (10%) are $CD56^{bright}$ and $CD16^{dim/neg}$. These NK cell subsets are phenotypically and functionally distinct. In addition, other NK cell subsets have been identified according to their tissue distribution. These cells have a different repertoire and effector functions compared to PB NK cells. These NK cell subsets are described in section 1.4.8.1.

1.3.1 $CD56^{bright}$ Natural Killer cells

NK cell development has been considered a linear model in which haematopoietic stem cells (HSCs) commit to the NK cell lineage and acquire NK cell receptors before becoming a fully functional $CD56^{dim}$ mature NK cell. It has been proposed that $CD56^{bright}$ cells are immature NK cells that will develop into $CD56^{dim}$ cells. Some evidence supports this observation, for instance during NK cell development *in vitro* or

even after HSCT, the first subset to arise is the $CD56^{bright}$ (Grzywacz *et al.*, 2006; Dulphy *et al.*, 2008). In addition, PB $CD56^{dim}$ cells display shorter telomeres compared to $CD56^{bright}$ cells from PB and lymph nodes (LNs) (Romagnani *et al.*, 2007). Although the notion of two different terminally differentiated cell populations is also a conceivable scenario. It has been suggested that $CD56^{bright}$ NK cells are indistinguishable from mature NK cells ($CD56^{dim}$) activated with IL-12, suggesting that $CD56^{bright}$ NK cells are not progenitors of the $CD56^{dim}$ subset but different populations (Loza and Perussia, 2004a).

$CD56^{bright}$ cells are known for their capacity to produce and secrete cytokines, such as granulocyte–macrophage colony-stimulating factor (GM-CSF), IFN-γ, interleukin (IL)-10, IL-13 and TNF-β. In addition, resting $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets show differences in their NK cell receptor repertoires (Voss *et al.*, 1998). $CD56^{bright}$ NK cells are characterised by the expression of the high/intermediate affinity IL-2 receptor, that confers the capacity to expand *in vitro* and *in vivo* in response to low doses of IL-2 (Caligiuri *et al.*, 1990; Caligiuri *et al.*, 1993). $CD56^{bright}$ NK cells do not express CD16 and express low levels of CD69, Killer-cell immunoglobulin-like receptors (KIRs) and intracellular perforin (Ferlazzo *et al.*, 2004b). In terms of distribution, $CD56^{bright}$ NK cells are dominant in LNs (around 75%). Additionally, the CCR7 LNs homing receptor is only expressed by $CD56^{bright}$ NK cells. $CD56^{bright}$ NK cells are capable of cytokine secretion but resting cells have low cytotoxicity. Nevertheless, after activation with IL-2 or IL-12, $CD56^{bright}$ cells exhibit similar or enhanced cytotoxicity against NK cell targets compared to $CD56^{dim}$ cells. Figure 1.3 shows the phenotype and effector functions of $CD56^{bright}$ cells in PB.

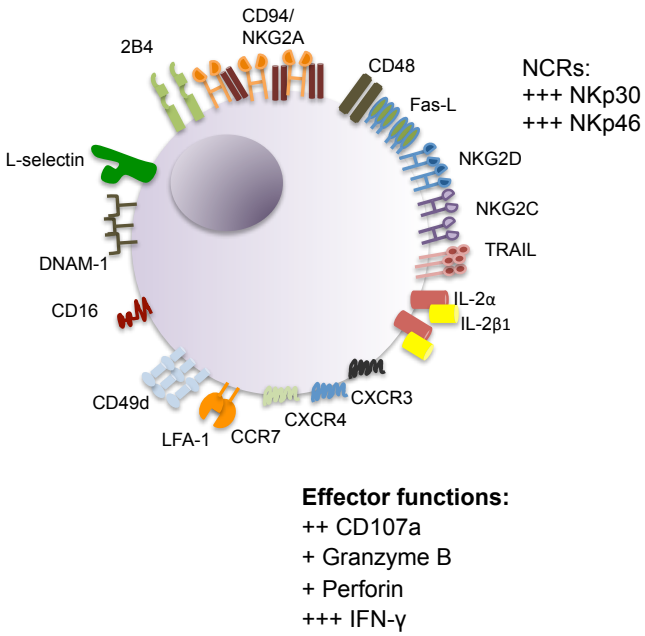


Figure 1.3. Phenotype of CD56^{bright} NK cell subset. CD56^{bright} NK cells express CD94/NKG2A, CCR7 and L-selectin, but lack KIRs and have low or no CD16 expression. CD56^{bright} NK cells are characterised for its abundant cytokine secretion.

1.3.2 CD56^{dim} Natural Killer cells

CD56^{dim} NK cells are considered to a terminally differentiated and mature NK cell subset. CD56^{dim} NK cells are cytolytic and function as efficient effectors of natural and antibody-dependent target cell lysis. In contrast to CD56^{bright} NK cells, resting CD56^{dim} NK cells express only the intermediate-affinity IL-2 receptor and proliferate weakly in response to high doses of IL-2 *in vitro* (Baume *et al.*, 1992). An important feature of CD56^{dim} NK cells is the expression of KIRs and CD16 but not CCR7 and L-selectin. CD56^{dim} cells are enriched in the bone marrow (BM), blood and spleen (Fehniger *et al.*, 2003; Ferlazzo *et al.*, 2004b; Freud *et al.*, 2006). CD56^{dim} cells express perforin and granzymes and are more cytotoxic against NK cell-sensitive targets (K562 and COLO205 cell lines) than CD56^{bright} NK cells (Nagler *et al.*, 1989). Figure 1.4 shows the phenotype and functions of CD56^{dim} NK cells.

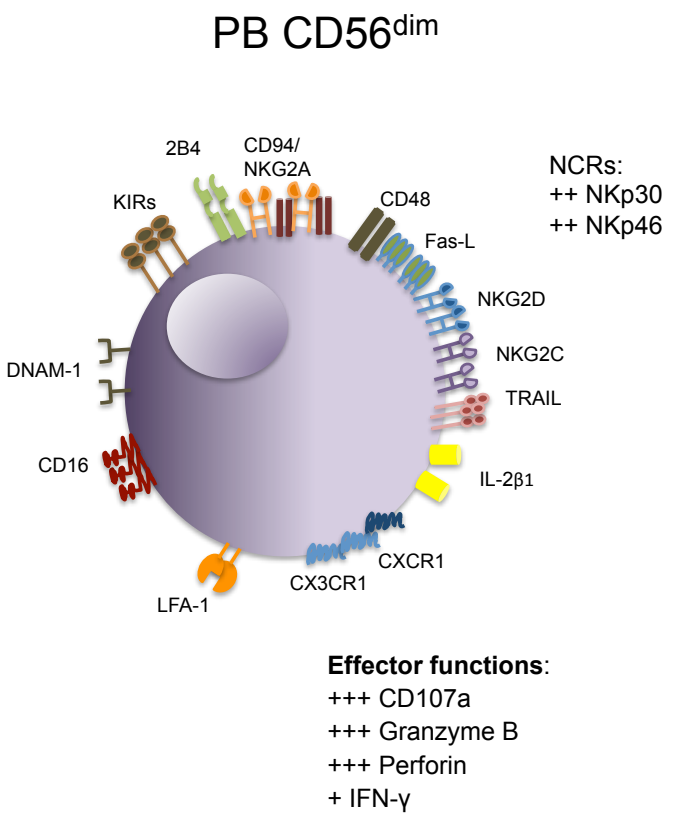


Figure 1.4. Phenotype of the CD56^{dim} NK cell subset. CD56^{dim} NK cells express KIRs and CD16, lack CCR7 and L-selectin expression. CD56^{dim} NK cells are characterised for its cytotoxic effector functions, expressing high levels of perforin and granzymes.

1.4 Natural Killer cell development

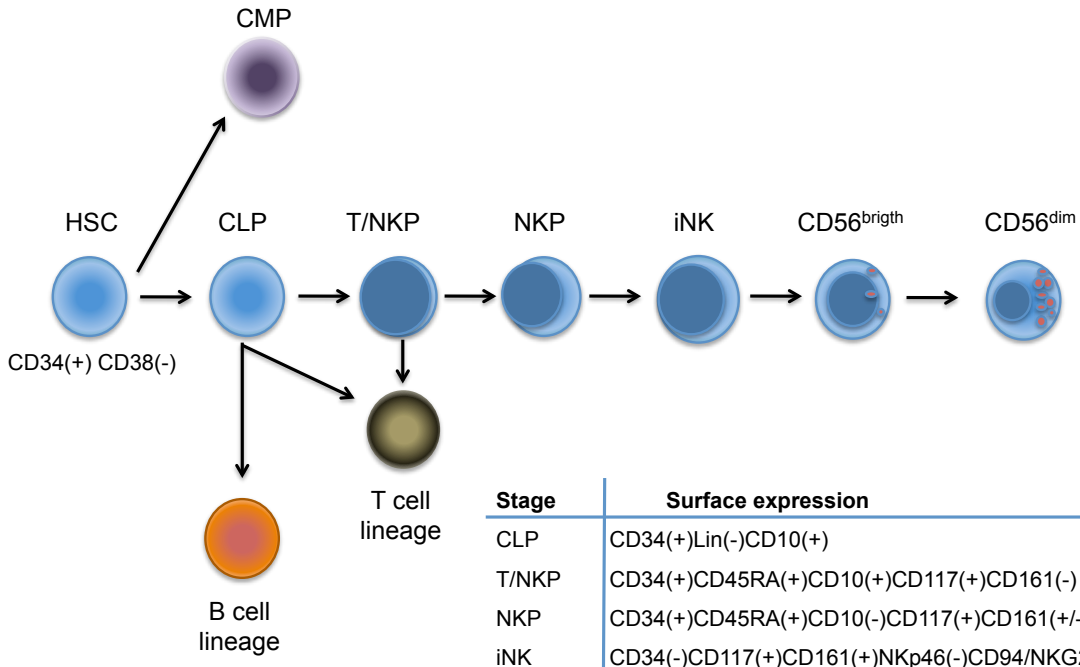
1.4.1 Natural Killer cell development in the bone marrow

HSCs have the potential to self-renew and proliferate without losing multipotency and ultimately to differentiate into all blood cell types. HSCs can be found in the liver during foetal life, but towards the end of the first semester HSCs populate the BM. In adults, HSCs reside mainly in the BM, where specialised stromal cells provide the specific microenvironment for self-renewal or differentiation (Orkin and Zon, 2008). During differentiation, the expression or repression of specific genes allows commitment towards a specific lineage.

Traditionally, haematopoiesis has been described as a process in which multipotent HSCs commit to either the myeloid-erythroid lineage or the lymphoid lineage. In theory, common lymphoid precursors (CLPs) do not generate myeloid cells and common myeloid precursors (CMPs) do not hold the potential to generate lymphoid cells (Akashi

et al., 2000). The controversy of the myeloid or lymphoid origin of NK cells has been disputed for a long time (Ortaldo and Herberman, 1984). Two decades ago, Sanchez et al. demonstrated that NK cells are closely related to T cells by the discovery of a T/NK cell precursor in humans (Sanchez *et al.*, 1994). Later experiments in mice allowed the identification of a CLP that was able to generate NK cells as well as B and T cells (Kondo *et al.*, 1997). Additional reports in humans have described the phenotype of the earliest NK cell progenitor using either BM or umbilical cord blood (CB) HSCs (Miller *et al.*, 1994; Miller *et al.*, 1998; Haddad *et al.*, 2004). Thus, it has been widely accepted that NK cells originate from lymphoid precursors. This model has been questioned recently by the observation of progenitors that preserve lymphoid and myeloid potential and lack erythroid potential (Katsura, 2002). In addition, recent reports have demonstrated that NK cells can also be derived from myeloid precursors cells (CMPs, granulo-monocytic precursors as well as $CD33^{+}CD13^{+}$ and macrophage colony-stimulating factor progeny) *in vitro* (Perez *et al.*, 2003; Grzywacz *et al.*, 2011).

Based on studies in mice, it was discovered that a natural killer cell precursor (NKP) was formed in the BM and developed into a mature NK cell. Nowadays, it is recognised that human NK cell development is a complex process that is still not fully understood. NK cells are derived from $CD34^{+}$ HSCs. HSCs give rise to CLP that further differentiate into T/NKP cells able to give rise to NKP and later immature NK (iNK) and then mature NK (mNK) cells as illustrated in figure 1.5 (Huntington *et al.*, 2007).



Stage	Surface expression
CLP	CD34(+)Lin(-)CD10(+)
T/NKP	CD34(+)CD45RA(+)CD10(+)CD117(+)CD161(-)
NKP	CD34(+)CD45RA(+)CD10(-)CD117(+)CD161(+/-)
iNK	CD34(-)CD117(+)CD161(+)NKp46(-)CD94/NKG2A(-)
$CD56^{bright}$	CD117(+/-) NKp46(+)CD94/NKG2A(+)KIR(+/-)
$CD56^{dim}$	CD117(-) NKp46(+)CD94/NKG2A(+/-)CD16(+)KIR(+)

Figure 1.5. NK cell development. NK cells are derived from HSCs and capable of differentiation into CLP and CMP lineages. CLP give rise to T, B and NK cells and further differentiate into T/NKP, NKP, iNK, $CD56^{bright}$ and finally $CD56^{dim}$ NK cells. The surface expression of specific cell surface antigens for each stage is shown on the right bottom corner.

Studies in mice identified 5 stages in NK cell development (Kim *et al.*, 2002). However, due to differences in phenotypic markers between human and mouse NK cells, human NK cell development studies cannot be directly correlated to murine studies. Therefore, the majority of the data involving human NK cell development has been achieved through HSCs culture *in vitro* using supporting feeder layers.

1.4.1.1 Common lymphoid progenitors

During haematopoiesis, HSCs differentiate into CMPs or into CLPs. CLPs are able to differentiate into B, T and NK cells. CLPs will further give rise to a T/NK progenitor that will subsequently differentiate into iNK cells and lastly mNK cells (figure 1.5). Although it was thought that haematopoiesis follows a strict pathway from pluripotent (HSCs) to oligopotent (CLPs or CMPs) and then to monopotent progenitors (Graf, 2002), recent studies have shed light into the plasticity of these cells (Grzywacz *et al.*, 2011). The following NK cell development description follows the traditional view of NK cells derived from CLPs.

A subpopulation of $Lin^-CD34^+CD38^+CD10^+$ cells has previously been described as CLPs able to give rise to B, NK and T cells but not to cells of the myeloid lineage (Galy *et al.*, 1995). CLPs undergo differentiation towards a NK/T progenitor. Some studies using CB or BM HSCs have suggested that the expression of CD7 or CD10 defines NK/T and B cell precursors, respectively (Miller *et al.*, 1994; Haddad *et al.*, 2004). Although more reports suggest that $Lin^-CD34^+CD10^+$ cells are restricted to the B cell lineage (Ryan *et al.*, 1997; Rossi *et al.*, 2003), CD7 or CD10 does not strictly identifiy a homogenous precursor population.

1.4.1.2 Natural Killer cell precursors, immature Natural Killer cells and mature Natural Killer cells

T/NKP cells commit to NKPs through a transition characterised by the expression of CD122 (IL-2/15Rβ subunit) (Ikawa *et al.*, 1999). The expression of CD122 confers IL-15 responsiveness, a cytokine that has been described to be key for NK cell development and maturation (Huntington *et al.*, 2009). NKPs cannot give rise to any other cell lineage, but NK cells. Next, NKPs differentiate into iNK cells. A population of "immature" NK cells ($CD3^-CD161^+CD56^-$) can be obtained *in vitro* by the culture of Lin^-CD34^+ CB cells (Bennett *et al.*, 1996), suggesting that CD161 identifies iNK cells (Lanier *et al.*, 1994). iNK cells are characterised by the expression CD2 and 2B4 (CD244) (Bennett *et al.*, 1996; Yu *et al.*, 1998; Sivori *et al.*, 2002). Furthermore, iNK cells do not express perforin and granzyme B (Grzywacz *et al.*, 2006) and display low cytolotytic activity, however further culture with IL-2 and IL-12 enhanced their cytotoxicity and CD56 acquisition (Bennett *et al.*, 1996; Zamai *et al.*, 1998b). iNK cells mature as acquisition of activation and inhibitory receptors occurs. The expression of activating markers occurs prior to the appearance of HLA class I, raising the question of what mechanisms are controlling auto-reactivity? A recent suggestion to explain these mechanisms is the expression of 2B4. It was reported that 2B4 expression occurs early during NK cell development and acts as an inhibitory receptor, avoiding the killing of autologous cells (Sivori *et al.*, 2002). The acquisition of receptor and of effector functions during NK cell development is a complex process still not well understood. The study of Grzywacz et al. used CD117 and CD94 to distinguish between iNK and mNK cells using a differentiation model *in vitro* (Grzywacz *et al.*, 2006). iNK cells ($CD56^+CD117^{high}CD94^-$) gave rise to mNK cells ($CD56^+CD117^{low}CD94^+$), the transition was associated with the acquisition of activating receptors NKp30, NKp46 and NKG2D, the inhibitory receptor NKG2A and functionality (cytotoxicity and IFN-γ production).

1.4.2 Natural Killer cell licensing/education

NK cells undergo a final maturation step through the recognition of self-molecules. A prototypical example of this process is the MHC class I-induced NK cell "education" or 'licensing". It has been suggested that NK cells undergo an "education" process to become functionally competent and also to recognise "self" and "non-self". This process requires the recognition of self-MHC class I molecules. The functional NK cell maturation process includes the licensing, also referred as education, arming or tuning, in which NK cells acquire functional competence after recognition of self-MHC class I molecules. A summary of these models is presented below.

1.4.2.1 Licensing model

In 2005, Yokohama and Kim introduced the "licensing" concept, commonly used synonymously as "arming". It was demonstrated in mice that NK cells that expressed self-inhibitory receptors ("licensed") produced more IFN-γ compared to those that did not express self-inhibitory receptors ("unlicensed"). The licensed cells can be inhibited by the same self-MHC class I that initially licensed them while the unlicensed do not need to be inhibited by MHC class I as they are not functionally competent (figure 1.6) (Kim *et al.*, 2005b; Yokoyama and Kim, 2006). These publications suggest that inhibitory receptors might have a dual role, transmitting activating signals during NK cell development and inhibitory signals in mature NK cells.

Although experiments in mice have been able to prove the "licensing/arming" theory, translating this knowledge to human systems is extremely difficult. Some efforts have been made in this regard, as recent studies confirmed the "licensing" mechanism in human NK cells (Kim *et al.*, 2008). For instance, human NK cells lacking the expression of self-inhibitory receptors (KIRs or NKG2A) were hyporesponsive in terms of cytokine secretion and cytotoxicity. On the contrary, NK cells with self-specific inhibitory receptors were fully functional (Anfossi *et al.*, 2006). Additionally, it was recently reported that infection with human cytomegalovirus (HCMV) promotes the differentiation and expansion of NK cells with self-specific inhibitory KIRs (Beziat *et al.*, 2013). Subjects that were not infected with HCMV had a random KIR repertoire, highlighting the effect of HCMV infection on the KIR repertoire (Beziat *et al.*, 2013).

Arming model

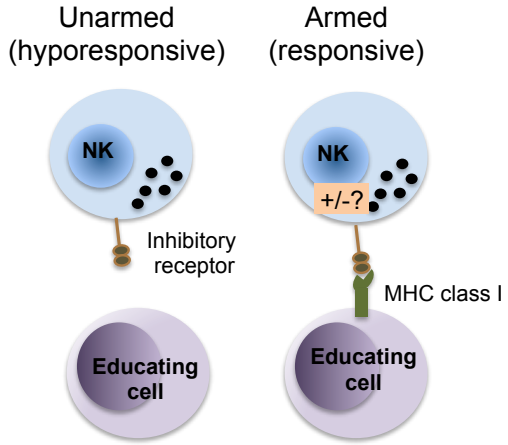


Figure 1.6. The licensing model. In the licensing or arming model, NK cells need the presence of an inhibitory receptor to become functional. In the absence of "arming", NK cells remain hyporesponsive. Adapted from Höglund and Brodin, 2010 with permission from Nature Publishing Group.

1.4.2.2 The education or disarming model

In 2005, Fernandez *et al*. showed that a population without self-inhibitory receptors in mice existed (Fernandez *et al.*, 2005). In contrary to the common belief that NK cell self-tolerance was achieved due to the expression of inhibitory receptor specific for MHC class I, these cells were self-tolerant and hyporesponsive (Fernandez *et al.*, 2005). Based on these results, the "disarming" model was proposed (Raulet and Vance, 2006; Joncker and Raulet, 2008). In summary, the balance of the stimulatory and inhibitory signals along with the presence of self-cells as NK cell develop will dictate the subsequent responsiveness of the mature NK cell (figure 1.7). In this context, if NK cells encounter cells with activating ligands and no MHC class I, NK cells are rapidly activated. However, in the case of prolonged stimulation, NK cell desensitisation occurs rendering them unable to interact with other cells. Thus, in the absence of host MHC class I, the interaction between NK cells and cells expressing activating ligands and no MHC class I also results in desensitisation.

Disarming model

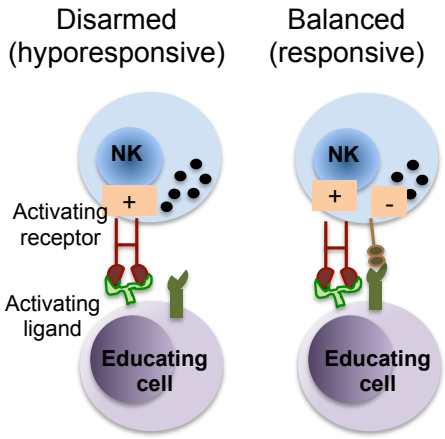


Figure 1.7. Disarming model of NK cell education. On the left side of the figure, the disarming model is presented. NK cells are fully functional by default, however, in the absence of self-inhibitory MHC class I, NK cells become hyporesponsive. But in the presence of self-inhibitory MHC class I, NK cells become responsive. In the Arming model (right side), NK cells need the presence of an inhibitory receptor to become functional. In the absence of "arming", NK cells remain hyporesponsive. Adapted from Höglund and Brodin, 2010 with permission from Nature Publishing Group.

1.4.2.3 Cis-interaction model

A property of inhibitory receptors is their ability to interact in *cis* with their ligand, that is, at the same membrane (Doucey *et al.*, 2004). The basis for this model is that Ly49 receptors in mouse can transmit inhibitory signals even when not engaged but only if present in the immunological synapse. In the cis-interaction model, the interaction between Ly49 receptor and MHC class I in *cis* sequesters Ly49, inhibiting its relocation to the immunological synapse. Therefore, the inhibitory signals of the unengaged Ly49 are absent and NK cells become more responsive (Chalifour *et al.*, 2009).

1.4.2.4 Rheostat model

It is known that NK cells express inhibitory receptors with various affinities. Thus, it is hard to conceptualise that NK cell responsiveness will depend on the expression of self-MHC class I based on the licensing and education models. The question arises as to whether NK cells exist either in hyper-responsive or hypo-responsive homogenous sets or if they would vary in their responsiveness. A previous report showed a direct correlation between the expression of MHC class I molecules and the capacity for

missing-self recognition (Johansson *et al.*, 2005) suggesting that NK cell responsiveness is influenced by the number of inhibitory receptors (Ly49 or NKG2A) able to engage self MHC class I molecules. The signals needed to achieve a response vary continuously in the Rheostat model (figure 1.8). NK cells can be tuned to a point in which a balance of inhibitory and activating signals is set in healthy cells. If NK cells lack self-MHC class I, the responsiveness will be adjusted downward below the threshold needed to attack healthy cells. If the NK cell expresses one inhibitory receptor for self-MHC class I, the threshold will be adjusted to a higher point, and even higher if the cell expresses two or more sets of inhibitory receptor for self-MHC class I (Joncker and Raulet, 2008). Hence, NK cell responses can be tuned "up" or "down" in a quantitative manner instead of a binary system (Brodin *et al.*, 2009).

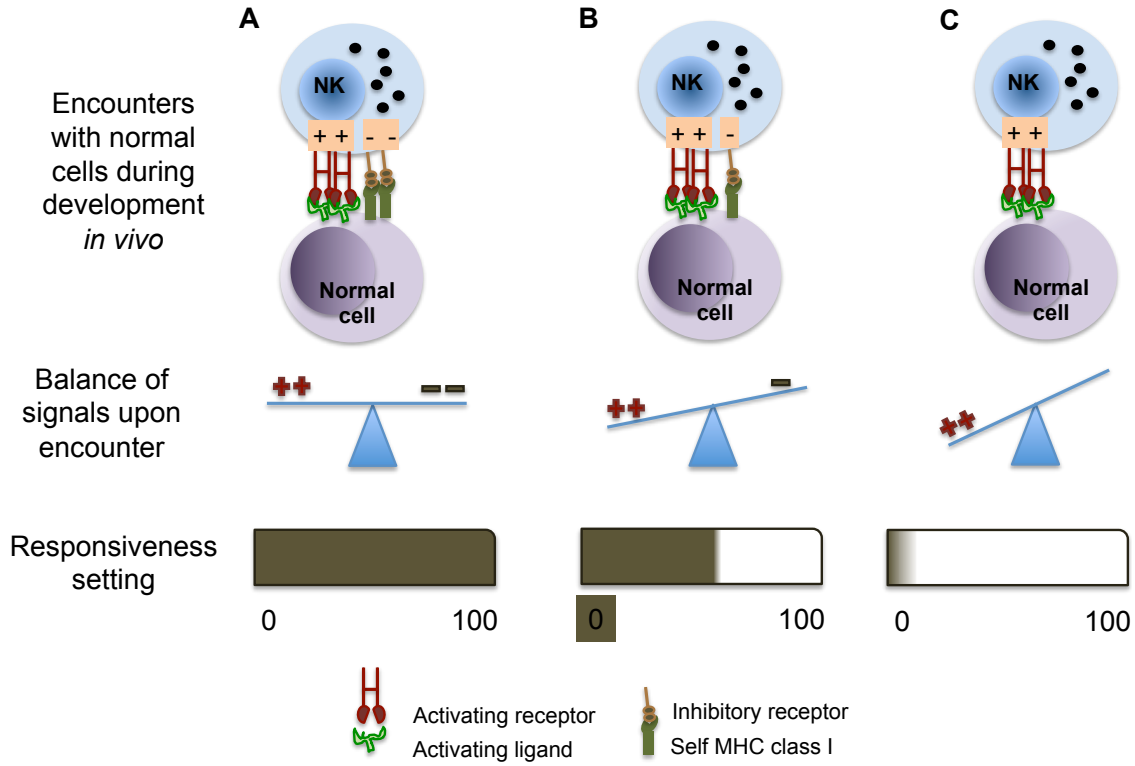


Figure 1.8. The Rheostat model. The tuning of NK cells is achieved during development, when cells are exposed to inhibitory and stimulatory interactions with self-cells. The balance of signals upon encounter with normal cells sets the responsiveness state of NK cells. A) A state of high responsiveness is acquired through the interaction of NK cells expressing several inhibitory receptors with normal cells expressing stimulatory signals. B) The partial hyporesponsiveness results from the encounter of NK cells expressing only one inhibitory receptor with self-cells expressing stimulatory signals. C) If no inhibitory receptors are expressed, the strong continuous stimulation induces hyporesponsiveness. Therefore, the NK cell responsiveness is tuned in a quantitative manner by the level of expression of inhibitory and stimulatory receptors on the cells in contact with NK cells during their development. (Joncker and Raulet, 2008). Copyright 2009, The American Association of Immunologist, Inc.

1.4.3 Cytokines required for Natural Killer cell development

Decades ago, very little was known about NK cell development and its requirements. $CD34^+$ HSC were cultured over BM stroma with the addition of IL-2 $CD56^+CD3^-$ that were phenotypically and functionally nearly identical to peripheral NK cells could be obtained in five weeks (Miller *et al.*, 1994). But, was IL-2 the key cytokine for NK cell development *in vivo*? This question arose because IL-2 is mostly available when T cells are activated and it was difficult to imagine that millions of NK cells were produced daily *in vivo* knowing that T cells would not be constantly producing IL-2. Effectively, it was shown later that NK cells were present in IL-2 and IL-2Rα knockout mice (Sadlack *et al.*, 1995; Willerford *et al.*, 1995); however, IL-2Rβ and IL-2γc knockout mice lacked NK cells (DiSanto *et al.*, 1995; Suzuki *et al.*, 1997). These results suggested that not IL-2 but a cytokine that binds to IL-2Rβ and IL-2γc was needed for NK cell development *in vivo*. Later, it was discovered that this cytokine was IL-15, as IL-$15^{-/-}$ and IL-$15R\alpha^{-/-}$ mice lacked NK cells (Lodolce *et al.*, 1998; Kennedy *et al.*, 2000).

Further experiments demonstrated that the culture of HSC with IL-15 gave rise to NK cells, however regardless of the high purity, only $CD56^{bright}$ cells and very low numbers were produced. These observations prompted the idea that additional cytokines were needed to produce high numbers of NK cells *in vivo* and raised the argument that $CD56^{bright}$ cells were precursors of $CD56^{dim}$ cells. The work by Matos *et al*. investigated the expression of c-kit receptor (CD117) on peripheral $CD56^{bright}$ cells and $CD56^{dim}$ cells, and found that about 50% of $CD56^{bright}$ cells expressed c-kit, a receptor also expressed in $CD34^+$ cells (Matos *et al.*, 1993). The use of c-kit ligand (SCF, stem cell factor) and FLT3-L (produced by BM stroma cells) demonstrated that increased NK cell numbers could be obtained when these factors were used together with IL-15 *in vitro* (Yu *et al.*, 1998). The mechanism through which SCF and FLT3-L increase NK cell numbers and aid NK cell development is by the induction of the expression of IL-2/15Rβ receptor on $CD34^+$ cells, making $CD34^+$ cells IL-15 responsive (Yu *et al.*, 1998). In summary, NK cell development is aided by the action of early acting cytokines (FLT3-L and SCF) and late acting cytokines (IL-15).

1.4.4 Transcription factors involved in Natural Killer cell development

NK cell development is finely orchestrated by molecular events that lead to the activation or repression of transcription factors (TFs). These TFs are able to bind to promoter regions in the DNA and regulate gene transcription. Depending on the stimuli,

some TFs are expressed or repressed, the balance of these events leads to the differentiation towards a certain cell lineage.

Although the expression of TFs in human NK cell development *in vitro* has recently been analysed (Pinho *et al.*, 2012), the majority of the data has been obtained using knockout mice. The following section includes the studies of TFs using knockout mice, however it is indicated when studies of TFs in humans have been performed.

The expression of TFs controlling NK cell development cannot be seen as a linear sequence of events, instead, it is a complex process in which interaction between these TFs occurs and different stimuli affect their expression and thus cell fate. For the purpose of simplifying the data obtained from murine models, the TFs described in this section are divided into three groups: 1) TFs involved in NK cell commitment, 2) TFs involved in NK cell differentiation and maturation and 3) TFs involved in regulation of NK cell effector functions (figure 1.9). Although a sequential approach was intended during this description, some of these TFs play a role early in NK cell development and also have an impact on NK cell effector functions.

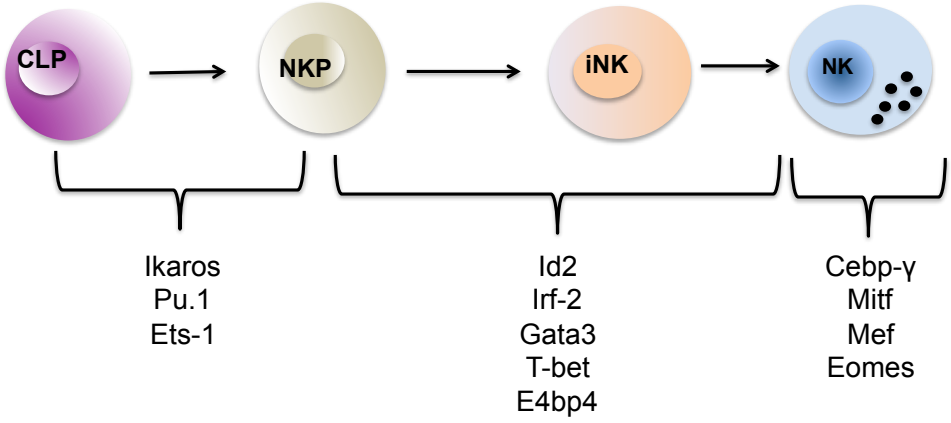


Figure 1.9. Transcriptions factors involved in NK cell development. The figure shows the expression of TFs divided into three groups. The first group acts on NK cell commitment, the second on NK cell maturation and the last one on NK cell effector functions.

1.4.4.1 Transcription factors involved in Natural Killer cell commitment

In the first group, the TFs Ikaros, Pu.1 and Ets-1 are included (Barton *et al.*, 1998; Boggs *et al.*, 1998; Colucci *et al.*, 2001). It has been shown that NKPs are reduced in mice lacking Ikaros, Pu.1 or Ets-1. Ikaros is a zinc finger protein able to bind the promoter of CD3 delta, initially described as an important TF for T lymphocyte development (Georgopoulos *et al.*, 1992) and later on for NK cell and B cell

development (Georgopoulos *et al.*, 1994). This TF might be important for the commitment of HSCs into CLPs. Ikaros knockout mice have reduced number of NK, T and B cells (Boggs *et al.*, 1998). Similarly, Pu.1 is essential for T and B cell development and to a lesser extent for NK cell development. Pu.1 knockout mice have lower numbers of mature NK cells which have a decreased response to cytokines (IL-2 and IL-12) (Colucci *et al.*, 2001). In progenitor cells, Pu.1 is needed for CD45 expression, suggesting that Pu.1 might also regulate CD45 expression in NK cells. NK cells deficient in CD45 are functionally defective, and resemble Pu.1 deficient NK cells (Huntington *et al.*, 2005; Hesslein *et al.*, 2006). Ets-1, a helix-turn-helix TF belongs to the Ets family, it also regulates NK cell development and functions. Ets-1 Knockout mice have a reduced number of NK cells in the spleen and BM (Barton *et al.*, 1998). Ets-1 participates in the regulation of other TFs such as T-bet and Id2 (Ramirez *et al.*, 2012) and plays a role in the induction of the IL-15Rβ (CD122) chain, required for IL-15 signalling (Ye *et al.*, 2005).

1.4.4.2 Transcription factors involved in Natural Killer cell differentiation and maturation

Once NKPs are generated, another set of TFs is needed for the subsequent steps leading to NK cell maturation, including Id2, Irf-2, Gata3 and T-bet. Id proteins act as transcriptional repressors that bind and antagonise the activity of E proteins that associate to specific DNA sequences (Sun *et al.*, 1991). Id proteins are essential for NK cell development and maturation; mice lacking Id2 have a reduced number of mNK cells in the spleen (Yokota *et al.*, 1999). Further analysis demonstrated that even though a decrease in mNK cell numbers was observed, there was no effect on the numbers of iNK cells (Boos *et al.*, 2007). Since NKP numbers were normal in $Id2^{-/-}$ mice, it was suggested that Id3 might compensate for Id2 giving rise to normal numbers of NKPs (Ramirez and Kee, 2010). The overexpression of Id3 in $CD34^{+}$ precursors cells was able to inhibit T cell development and favoured NK cell development (Heemskerk *et al.*, 1997).

In the absence of Irf-2, Gata3 and T-bet, NK cells are generated, but they are reduced in numbers and exhibit an immature phenotype and reduced effector functions (Samson *et al.*, 2003). Members of the interferon regulatory factor (Irf) have been previously described to bind DNA sequences common to the promoters of IFN-α and IFN-β. Irf-2 binds to a specific sequence in the IFN-β gene and acts as a transcriptional repressor (Harada *et al.*, 1990). $Irf\text{-}2^{-/-}$ mice have decreased mNK cells, but the liver and spleen contain normal numbers of iNK cells (Taki *et al.*, 2005). NK cells from Irf-2

knockout mice had reduced cytotoxicity and lower production of IFN-γ (Lohoff *et al.*, 2000). Similarly, NK cells from Gata3$^{-/-}$ mice exhibited immature phenotype and were poor producers of IFN-γ (Samson *et al.*, 2003). An interesting observation was that BM NK cells from Gata3$^{-/-}$ mice were not able to migrate to the liver (Samson *et al.*, 2003). T-bet and H2.0-like homeobox (Hlx) were described to be transcriptional targets of Gata3, providing a possible explanation for the similar phenotype observed in T-bet$^{-/-}$ mice (Samson *et al.*, 2003). T-bet$^{-/-}$ mice have reduced numbers of NK cells in the PB, spleen and liver, but NK cell accumulation in LNs and BM showed an immature phenotype (Townsend *et al.*, 2004; Jenne *et al.*, 2009). In addition to the immature phenotype, NK cells had low cytotoxic and cytokine production potential and decreased longevity (Townsend *et al.*, 2004; Werneck *et al.*, 2008).

Another transcription factor studied in mice, Gata2, has been reported to be important for the maintenance of the HSCs pool (Tsai and Orkin, 1997). However, there is actually no study linking this TF to NK cell development. In humans, the analysis of patients with GATA2-deficiency showed decreased NK cell numbers in the periphery, in particular of CD56^{bright} NK cells. During this study, it was demonstrated that GATA2 is necessary for the presence of CD56^{bright} NK cells and is necessary for the functional maturation of CD56^{dim} NK cells, suggesting that this TF plays a key role during NK cell maturation (Mace *et al.*, 2013).

There are some TFs that are crucial for the differentiation of certain lineages; these are called master regulators. For instance, Pax5 is a master regulator for B cell development in mice. Cells lacking Pax5 are not able to differentiate into B cells, but retain the ability to differentiate into T or NK cells (Schaniel *et al.*, 2002). Notch1 and Gata3 play a similar role supporting T cell development (Pear and Radtke, 2003; Maillard *et al.*, 2005). A similar TF was recently discovered for NK cell commitment, E4bp4. This TF was the only one described so far necessary for NK cell development. Using knockout mice for E4bp4, Gascoyne *et al*. showed that B, T and NKT cells were present in E4bp4-/- mice but observed a decrease in iNK cells numbers and a greater reduction of mNK cells (Gascoyne *et al.*, 2009). Gene expression analysis revealed that Gata3 and Id2 expression was reduced in the absence of E4bp4, indicating that both act downstream of E4bp4. Others found similar observations in mice (Kamizono *et al.*, 2009) and recent studies in humans confirm the exclusive expression of this TF by NK cells (Vacca *et al.*). E4bp4 plays a key role in the progression of NKPs to iNK cells and mNK cells.

1.4.4.3 Transcription factors involved in regulation of Natural Killer cell effector functions

Lastly, another set of TFs appears to regulate terminal maturation, NK cells lacking these TFs show normal phenotype but fail to exhibit normal cytotoxicity or cytokine expression. These TFs include CCAAT-enhancer binding protein (Cebp-g), microphthalmia transcription factor (Mitf), myeloid ELF1-like (Mef) and Eomes. Mice deficient in Cebp-g have normal numbers of T lymphocytes, B lymphocytes and NK cells, but NK cell cytotoxicity and IFN-γ production are impaired (Kaisho *et al.*, 1999). Similar observations have been reported in mice with a deficiency in Mitf, a regulator of melanocyte, osteoclast and mast cell differentiation and function (Cheli *et al.*, 2010). For studies of this TF, $Mitf^{mi}/Mitf^{mi}$ mice were generated with the mi mutant allele encoding an abnormal Mitf (Kataoka *et al.*, 2005). $Mitf^{mi}/Mitf^{mi}$ mice had normal numbers of NK cells, suggesting that this TF is not involved in NK cell development. However, the NK cells had impaired cytotoxicity and IFN-γ production in response to IL-12 and IL-18 stimulation (Ito *et al.*, 2001; Kataoka *et al.*, 2005), which was subsequently explained as due to a lack of perforin expression. It could be that $Mitf^{mi}$ interacts with Mef, preventing its entrance to the nucleus and obstructing further interaction with the perforin promoter (Lacorazza *et al.*, 2002). Mef is a member of the Ets family and like for Ets-1 Mef knockout mice have a reduced number of NK cells in the spleen. The few splenic NK cells are not able to produce IFN-γ and the remaining cells were able to bind cell targets but had low cytotoxicity (Lacorazza *et al.*, 2002). The observed lack of cytotoxicity was explained by the lack of perforin in NK cells. Similarly, Eomes has been reported to control perforin and also granzyme B expression in $CD8^+$ T cells (Intlekofer *et al.*, 2005) and was demonstrated to be important in maintaining mature NK cell attributes (Gordon *et al.*, 2012).

1.4.5 Natural Killer cell development sites

Knowledge to date indicates that NK cells derive from $CD34^+$ HSCs (Miller *et al.*, 1994), and that NK cell development occurs mainly within the BM microenvironment (Colucci *et al.*, 2003). However, a large and growing body of literature indicates that the site(s) of NK cell maturation might not be restricted to BM. HSCs can give rise to lymphoid precursors able to migrate to different sites that support NK cell differentiation.

NK cell development was originally thought to occur solely in the BM. Experiments in mice provided evidence supporting the important role of BM for NK cell development *in vivo* (Haller and Wigzell, 1977). However, LNs and secondary lymphoid tissues (SLT)

were relatively recently identified as sites of NK cell development due to their haematopoietic progenitor cell (HPCs) content (Freud and Caligiuri, 2006). The most accepted NK cell development model comes from this study, in which consecutive NK cell developmental stages were identified starting from LNs $CD34^+$ cells. Freud *et al*. found a $CD34^{+dim}CD45RA^+$ population expressing integrin α4β7 in LNs able to differentiate into NK cells in the presence of IL-15 (Freud *et al.*, 2005). The expression of integrin α4β7 has been previously linked to gut and LN homing (Yoshida *et al.*, 2001), thus this group suggested that $CD34^{low}Lin^-$integrinα4β7$^+CD117^{hi}CD56^-CD94^-CD16^-$ cells could migrate to LN and differentiate into $CD56^{bright}$ NK cells. Interestingly, one of the intermediates identified during the development process was $CD161^+CD117^{hi}CD94^-$, also found during the culture of CB $CD34^-lin^-$ cells (Grzywacz *et al.*, 2006). More importantly, these two $CD117^{hi}CD94^-$ populations were able to give rise to $CD56^+CD94^+$ cells *in vitro*, demonstrating the presence of NKP. This developmental model is based on the identification of four NK cell stages within LN: pro-NK cells (stage 1), pre-NK cells (stage 2), iNK cells (stage 3) and NK cells (stage 4) (figure 1.10). A final stage 5 ($CD56^{dim}$ cells) is proposed as the last step of NK cell maturation (Freud *et al.*, 2006). Although in this model CD56 acquisition is not a critical criterion, its expression increases as NK cell stages develop.

LNs are not known to be a HSC depository suggesting that NK cell lineage commitment is likely to take place somewhere else outside LNs. It has been proposed that the LN NKP population is derived from the BM, but recent data in mice suggests that the thymus might be also a NKP source (Veinotte *et al.*, 2008). It is possible that the LN microenvironment provides some of the signals and factors for NK cell development, including IL-15. As it is known that IL-15 is key for NK cell development, LNs must deliver this interleukin probably through the trans-presentation provided by dendritic cells or macrophages.

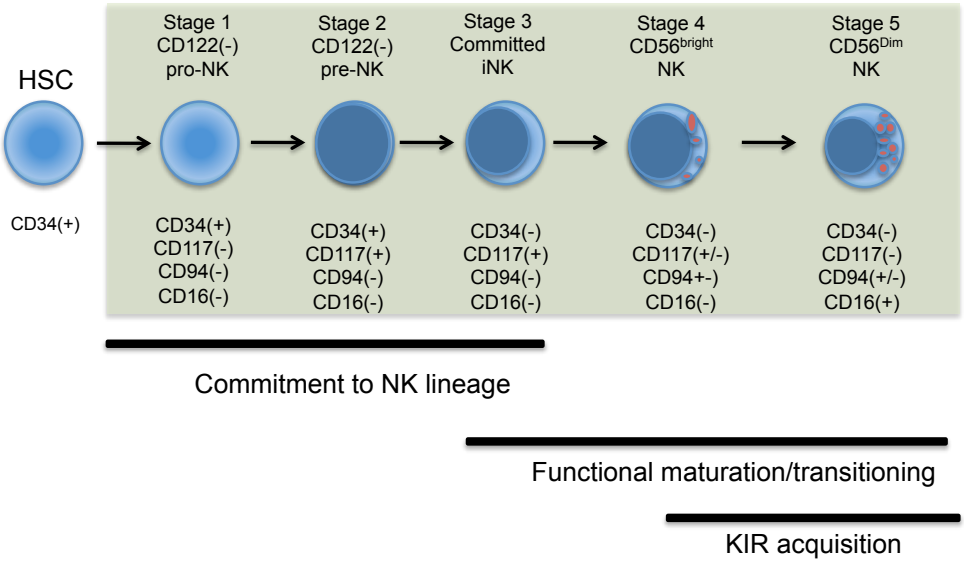


Figure 1.10. NK cell development in lymph nodes. The model proposed by Freud *et al*. suggests that NK cells derive from BM HSCs, the stages within the grey box may occur in LN. HSCs develop from pro-NK cells to pre-NK cells losing their capacity for T and B cell differentiation. From stages 3-5, NK cells mature through the acquisition of receptors and effector functions. $CD56^{bright}$ NK cells produce IFN-γ while $CD56^{dim}$ mediate cellular cytotoxicity. During the final steps, KIR acquisition is likely to occur. Freud and Caligiuri, 2006 (permission for reproduction granted by John Wiley and Sons).

Some years ago, a cell population in the foetal LN able to differentiate into NK cells and DC was described (Mebius *et al.*, 1997). Later, these cells were identified as lymphoid tissue inducer (LTi) cells, now known to play an important role in the organisation of LNs, intestinal cryopatches and Peyer's patchers (Eberl and Littman, 2003). Recently, it was demonstrated that foetal and adult LTi cells could differentiate into an atypical NK cell subset *in vitro* expressing $CD127^{+}$ and expressing RORC (Cupedo *et al.*, 2009). It has been reported that other tissues, such as the intestinal epithelial layer, also contain NKPs (Chinen *et al.*, 2007). The culture of these precursors with IL-15 gave rise to $CD56^{+}$ cells with less cytotoxic activity but higher IFN-γ production in comparison to PB NK cells.

Another possible site of NK cell development is the thymus. Vosshenrich *et al*. have described NK cell development in the thymus in mice (Vosshenrich *et al.*, 2006). Thymic-NK cell development results in NK cells with a distinct phenotype characterised by high expression of IL-7R ($CD127^{+}$) and the transcription factor GATA3. The thymic environment provides different signals or factors that shape NK cell phenotype and functions, highlighting the importance of the environment in NK cell development. In 2012, Hidalgo *et al*. identified human NK cell precursors in the thymus characterised by the expression of the TF BMPRIA. The culture of these precursors in the presence of SCF and IL-15 resulted in functional NK cells (Hidalgo *et al.*, 2012). Another interesting feature of thymic NK cells is the expression of intracellular CD3ε (iCD3ε), absent in

peripheral NK cells (De Smedt *et al.*, 2007). The significance of this expression is still unclear. Some authors have suggested that iCD3ε might arise due to the thymic microenvironment, including Notch ligands such as OP9-Delta-like 1 (DL1) (De Smedt *et al.*, 2007).

Finally, the membrane lining the uterus during pregnancy or maternal decidua has been shown to contain HPCs. These HPCs could differentiate *in vitro* into NK cells with a singular phenotype (Vacca *et al.*). Decidua NK cells (dNK) have a $CD56^{bright}CD16^{-}$ phenotype but show different functions compared to their counterpart in PB. Given the high density of CD56 expression, it has been thought that dNK cells derive from the $CD56^{bright}$ NK cell subset. However, there are some differences, the main ones being the expression of KIRs and CD69, which does not occur in $CD56^{bright}$ PB cells. In addition, dNK cells secrete vascular endothelial growth factor (VEGF), IL-8 and stromal-cell derived factor (SDF)-1 involved in angiogenesis and tissue remodelling.

Nonetheless, even with the evidence that NK cell development can occur at different sites, it is plausible that their presence in different tissues could be due to migration of NK cells from either the BM or PB. Shaping of the NK cell surface marker repertoire and functions by the environment may account for the differences observed between the different subsets.

1.4.5.1 Natural Killer cell subsets with specialised functions

1.4.5.1.1 Uterine Natural Killer cells

Uterine NK (uNK) cells comprise around 70% of the endometrial leukocytes found in the first trimester decidua (Bulmer *et al.*, 1991). The exact function of uNK cells remains largely unclear, but it seems they interact with the trophoblast; as a consequence, uNK cells release factors that modify the uterine spiral arteries (Hanna *et al.*, 2006). VEGF is important factor secreted by uNK cells (Moffett-King, 2002; Vacca *et al.*, 2011) that stimulates angiogenesis. uNK cells are characterised by their lack of expression of L-selectin, CD16 and CD57 while expressing KIRs and CD69 and CD56 (superbright). Additionally, the study of endometrial NK cells (eNK) and uNK cells revealed that pregnancy alters the KIR repertoire of uNK cells with bias towards HLA-C recognition (Male *et al.*, 2011). uNK cells have low cytolytic activity against K562 cells (Ferry *et al.*, 1990). Importantly, uNK cells have a different cytokine secretion profile (TNF-α, TGF-β, GM-CSF and G-CSF [granulocyte colony-stimulating factor] among others) compared to $CD56^{bright}$ NK cells (Saito *et al.*, 1993).

1.4.5.1.2 Liver Natural Killer cells

The liver is another organ in which up to 30-50% of the hepatic leucocytes present are NK cells. NK cells in the liver are involved in the regulation of hepatic immunity against pathogens. It has been shown that liver resident NK cells or blood-recruited NK cells have a distinct phenotype and function. The liver contains a 50/50 ratio of $CD56^{bright}$ and $CD56^{dim}$ NK cells. Liver $CD56^{dim}$ NK cells express KIR and NKG2A, and interaction with Kupffer cells can lead to cytolytic activity and cytokine production (Tu *et al.*, 2008; Burt *et al.*, 2009). Liver NK cells have high expression of CD69 and NKp44 and lower L-selectin expression with a decreased cytotoxic potential compared to PB NK cells (Burt *et al.*, 2009). The liver contains lower number of licensed NK cells compared to PB, thus the lower cytotoxicity of liver NK cells could be due to their unlicensed status (Burt *et al.*, 2009).

1.4.5.1.3 Thymic Natural Killer cells

Vosshenrich *et al*. described the development of thymic NK cells in mice (Vosshenrich *et al.*, 2006). This report included the characterisation of human thymic cells; $CD56^{hi}CD16^{-}$ cells that expressed CD127 (IL-7Rα) and the transcription factor GATA-3. $CD56^{hi}CD16^{-}$ cells produced cytokines but were less cytotoxic compared to their PB counterpart $CD56^{+}CD16^{+}CD127^{-}$. The thymic microenvironment likely has an important role in establishing this phenotype, however, the signals that regulate this process are still undefined.

1.4.5.1.4 NK-22 cells

NK cells can be found in healthy intestine mucosae, mainly within the lamina propria and in the tonsils or Peyer's patches (Cella *et al.*, 2009). These cells are characterised by the expression of NKp44, usually expressed in activated NK cells, and CCR6. NK-22 cells do not express perforin and little granzyme B. Moreover, NK-22 cells have very low intracellular IFN-γ but high IL-22 secretion, which is the hallmark of NK-22 cells.

A summary of NK cell subsets within different tissues is illustrated in figure 1.11.

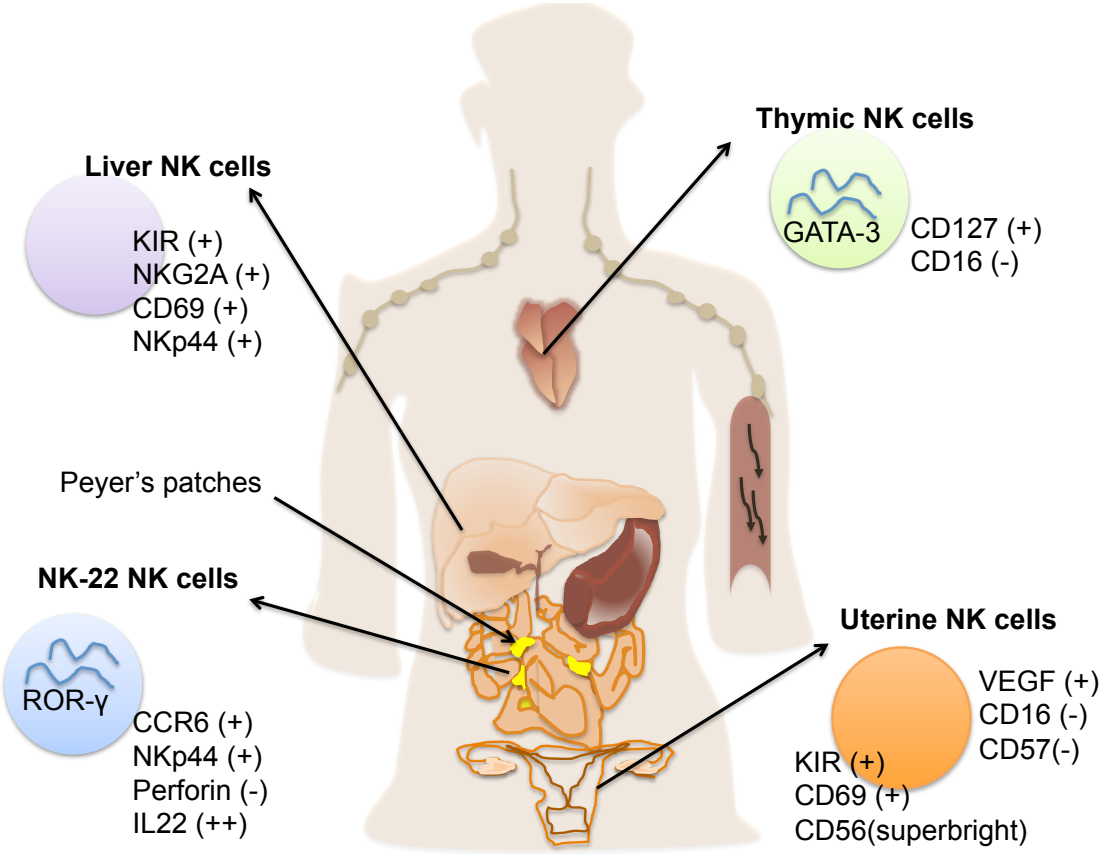


Figure 1.11. NK cell subset distribution. NK cells can be found in liver, thymus, peyer's patches and uterine decidua. The main features defining each subset are presented.

1.4.5.1.5 Memory Natural Killer cells?

Studies in mice suggest that NK cells can also have memory-like properties, similar to cells of the adaptive immune system (O'Leary *et al.*, 2006). In 2009, using a mouse model of murine cytomegalovirus (MCMV) infection, Sun *et al*. demonstrated that NK cells bearing the receptor Ly49H (MCMV-specific) were able to degranulate and produce cytokines after reactivation when these cells were transferred into naïve mice that were challenged with MCMV (Sun *et al.*, 2009). In the same year, the study by Cooper *et al*. suggested that murine NK cells could retain an intrinsic memory of prior activations (Cooper *et al.*, 2009). Later in 2010, a study from Paust *et al*. reported that adoptive transfer of virus-sensitised NK cells into naïve mice enhanced the survival of the mice after challenging with the sensitised virus but not with other virus. Only hepatic NK cells were able to develop specific memory and it was dependent on CXCR6 expression (Paust *et al.*, 2010). The latest study from Keppel and Cooper *et al*. proposed that cytokine activation of murine NK cells could generate memory-like NK cells able to produce IFN-γ. These cells once stimulated *in vitro* using cytokines or target cells had an intrinsic enhanced capacity to produce IFN-γ (Keppel *et al.*, 2013). Although strong evidence for adaptive features of murine NK cells has been provided,

studies of NK cell memory in humans are more complex but efforts to demonstrate similar behaviour have been made. Similar to the MCMV model, it has been observed after HSC transplantation (HSCT) the specific expansion of human NK cells expressing inhibitory receptors for ligands missing in the host (Ruggeri *et al.*, 1999; Ruggeri *et al.*, 2002). Strong evidence supporting the memory-like features of NK cells is clearly required and must address how long this "memory" persists. A recent report studying the serial killing by NK cells (cell line NK92) found that there was a short-term memory effect in which the first killing was slow but following events were faster (Choi and Mitchison, 2013). Further research in this area is likely to reveal more NK cell properties that may be useful for therapeutic purposes.

1.5 Natural Killer cell receptors

The surface marker repertoire acquired by NK cells is regulated by a dynamic process that is modulated by the environment. Unlike T and B cells, NK cells lack antigen-specificity whilst expressing a wide variety of activating and inhibitory receptors. The engagement of these receptors provides signals, which dictates whether an NK cell is activated or inhibited. NK cells are ready-to-go; therefore the regulation of this immediate response needs to be tightly regulated for the protection of healthy cells. In the following sections a description of the activating and inhibitory receptors expressed by NK cells is presented.

1.5.1 Activating receptors

For some time, it was thought that inhibition of NK cells was only controlled by KIRs recognising reduced expression of class I MHC (missing-self hypothesis). However, it has now been well established that the signalling downstream specific activating receptors is needed for NK cell activation and killing. The evidence for this came from the development of monoclonal antibodies able to block NK cell-mediated killing of malignant cells (Vitale *et al.*, 1998; Pende *et al.*, 1999). The activating NK cell receptors can be grouped into three categories: 1) receptors that signal through ITAM (immunoreceptor tyrosine-based activation motifs)-containing subunits, 2) the DAP10 associated receptor NKG2D and 3) other receptors that signal using different pathways.

1.5.1.1 ITAM bearing Natural Killer cell receptors

Many NK cells express receptors with ITAMs that induce a strong activation upon cross-linking. ITAMs are connected via non-covalent association of adaptor molecules such as DNAX-activating protein 12 kDa (DAP12), FcεR-γ or CD3ζ. These adaptor molecules contain an aspartic acid residue with a negative charge required for oligomer formation. DAP12 and FcεR-γ contain a single ITAM, and CD3ζ has three per chain (Lanier, 2008). The activation of the ITAM receptor induces reorganisation of the cytoskeleton, necessary for polarisation and release of cytotoxic granules, and the transcription of cytokines and chemokines. ITAM-bearing receptors include the natural cytotoxicity receptors (NCRs), CD94/NKG2 heterodimers, CD16 and activating KIRs.

NCRs

NCRs are NK cell-specific receptors involved in natural cytotoxicity against many targets without prior sensitisation. It has been reported that NCRs interact with viral proteins (Arnon *et al.*, 2005) and although their involvement in tumour cell killing is known (Pessino *et al.*, 1998; Pende *et al.*, 1999), most NCRs ligands still await identification. NKp30, NKp46 and NKp80 are expressed on resting and activated NK cells, whereas NKp44 is expressed only on activated NK cells (Moretta *et al.*, 2000). Known ligands for NCRs are described in table 1.1.

Table 1.1. NCR ligands. (Koch *et al.*, 2013). Reproduced with permission from Elsevier.

Receptor	Ligand	Source	Reference
NKp30	B7-H6	Tumour cells	(Brandt *et al.*, 2009)
	BAG6	Stressed cells, tumour cells	(Pogge von Strandmann *et al.*, 2007)
	Pp65	HCMV (negative effect)	(Arnon *et al.*, 2005)
	P*f*EMP1	*Plasmodium falciparum*	(Mavoungou *et al.*, 2007)
	Viral HA	Poxvirus, vaccinia virus (negative effect)	(Jarahian *et al.*, 2011)
	Heparin and heparin sulphate	All animal cells	(Ito *et al.*, 2012)
NKp44	Sialylated and sulfated proteoglycans	All animal cells	(Ito *et al.*, 2012)
	Viral HA and HN	Influenza virus, Sendai virus, Newcastle disease virus	(Jarahian *et al.*, 2009)
	Unknown	Bacterial cell wall components of mycobacteria and others	(Esin *et al.*, 2008)
	Heparin and heparin sulphate	All animal cells	(Hecht *et al.*, 2009)
NKp46	Unknown	*Fusobacterium nucleatum*	(Chaushu *et al.*, 2012)
	Viral HA and HN	Influenza virus, poxvirus, Sendai virus, Newcastle disease virus	(Arnon *et al.*, 2004; Glasner *et al.*, 2012)
	Unknown	Variety of normal and tumour cells	(Arnon *et al.*, 2006)
	Heparin and heparin sulphate	All animal cells	(Hecht *et al.*, 2009)

The first NCR discovered was NKp46 (Sivori *et al.*, 1997), expressed both in activating and resting NK cells but no other cell type. NKp46 activation is mediated through association with the adaptor molecules CD3ζ and FcεR-γ (Lanier, 2003). It is clear that NKp46 plays a key role during NK cell activation against various targets (Sivori *et al.*, 1999) although the identification of NKp46 ligands is still a challenge. It was suggested that heparan sulfate proteoglycans (HSP) were involved in the recognition of tumour cells by NK46 and NKp30 receptors (Bloushtain *et al.*, 2004) and absence of heparan sulfate expression reduced NK cell-mediated killing. Other ligands such as hemagglutinin molecules derived from influenza virus were also reported as ligands for NKp46 and NKp44 (Arnon *et al.*, 2001) (see table 1.1).

NKp44 was the next NCR discovered on human NK cells, revealed by blocking of this receptor partially inhibiting the killing of MHC-deficient targets (Vitale *et al.*, 1998). NKp44 signalling occurs via association with the adaptor molecule DAP12 and expression is restricted to activated cells *in vitro* (Vitale *et al.*, 1998). However *in vivo*, several NK cell subsets express NKp44, such as those found in human tonsils

(Ferlazzo *et al.*, 2004a) and dNK cells during pregnancy (Hanna *et al.*, 2006). Similar to NKp46, very little is known about NKP44 ligands although the influenza hemmaglutinin has been described earlier in addition to other viruses as potential ligands (see table 1.1) (Arnon *et al.*, 2001; Arnon *et al.*, 2004).

The third NCR discovered was NKp30, expressed in all human NK cells (Pende *et al.*, 1999). NKp30 may cooperate with NKp46 and NKp44 by triggering cytolysis through the transmembrane signalling molecule CD3ζ (Byrd *et al.*, 2007). It was reported that NKp30 recognises an unknown ligand on immature but not mature DCs (Cooper *et al.*, 2004). Hence, NKp30 is involved in the regulation of the immune response through interactions with DCs, resulting in their maturation (Pende *et al.*, 1999). A HCMV protein, pp65, was reported to interact with NKp30, antagonising NKp30 activation due to the dissociation of the adaptor molecule CD3ζ from NKp30 (Arnon *et al.*, 2005). As pp65 is an intracellular protein, it was proposed that soluble pp65 derived from the lysis of infected cells could bind NKp30 and block NK cell activity. Later, it was found that NKp30 could bind intracellular ligands such as HLA-B-associated transcript 3 (BAT-3), which is released from the tumour cells. BAT-3 binding to NKp30 mediated cytotoxicity, making BAT-3 the first cellular ligand identified for NKp30 (see table 1.1) (Pogge von Strandmann *et al.*, 2007).

CD94/NKG2 heterodimers

Another family of receptors is the C-type lectin family CD94/NKG2A/C/E. CD94 is a type II integral membrane glycoprotein that forms heterodimers with different members of the NKG2 family. These receptors are thought to be important for the recognition of HLA-E, the non-classical MHC class I molecule (Braud *et al.*, 1998). HLA-E presents peptides derived from other class I molecule leader peptides. The heterodimers CD94/NKG2C and CD94/NKGE associate with DAP12, and consequently play a role in NK cell activation (Borrego *et al.*, 1998; Braud *et al.*, 1998; Lee *et al.*, 1998). $NKG2C^{+}$ NK cells have been associated with HCMV infection and other diseases (Guma *et al.*, 2006a; Guma *et al.*, 2006b). In mice, NKG2E has been shown to be required for the protection of B6 mice against mousepox (ectromelia virus) (Fang *et al.*, 2011). It has been shown that dNK cells have poor cytotoxicity (Ferry *et al.*, 1990), however, during HCMV infection both NKG2C and NKG2E have been reported to be involved in the acquisition of cytotoxic functions thus controlling infection (Siewiera *et al.*, 2013).

CD16

Another activating receptor, CD16 (FcγRIIIa), couples with CD3ζ and with FcεR-γ (Moretta *et al.*, 2002). CD16 has low affinity for IgG receptor mediating ADCC, a process in which NK cells recognise the Fc portion of IgG bound to infected/transformed cells triggering cell death (Mandelboim *et al.*, 1999). CD16 is a potent activating receptor, being the only one able to elicit cytotoxicity alone without the need of other receptors (Bryceson *et al.*, 2006b).

Activating KIRs

Besides KIRs acting as inhibitory receptors, another set of KIRs was found to enhance NK cell cytotoxicity (Moretta *et al.*, 1995). They posses a short cytoplasmic tail without any signalling motif; however, they associate with the adaptor protein containing an ITAM-signalling motif DAP12 (Biassoni *et al.*, 1996). The ligands that associate with activating KIRs are poorly characterised, although major efforts have been made to define them. For instance, the study of KIR2DS4 on NK cells revealed interactions with MHC class I molecules and non-MHC class I molecules on melanomas enhancing NK cell cytotoxicity (Katz *et al.*, 2004).

1.5.1.2 NKG2D

NKG2D is expressed as a homodimer on almost all NK cells and does not heterodimerise with CD94. NKG2D associates with an adaptor molecule, DAP10, a subunit that carries a phosphatidylinositol-3 kinase (PI3K)-binding motif (Wu *et al.*, 1999). Some of NKG2D ligands are related to MHC class I molecules, usually expressed at low levels by normal cells but upregulated during cellular transformation or stress. This type of recognition, in which unhealthy cells upregulate self-ligands for activating receptors is called “induced self-recognition” (Diefenbach and Raulet, 2001), contrasting with “missing self-recognition”. Some reports show that NKG2D signals alone are sufficient to activate NK cells (Sutherland *et al.*, 2002; Lodoen and Lanier, 2005), while another study revealed co-stimulation with other receptors such as 2B4 was necessary (Bryceson *et al.*, 2006b).

NKG2D recognises MHC class I polypeptide-related chain A and B (MICA and MICB) and up to six different proteins named ULBPs (UL-16 binding proteins). Some of the ULBPs are transmembrane proteins (ULBP4-5) and others are glycophosphatidylinositol (GPI)-anchored proteins (ULBP1-3 and ULBP6) (Raulet *et*

al., 2013). Cellular stress plays a key role in the regulation of NKG2D ligands. Although "stress" is a very ambiguous term, some efforts in this area have shed light into the molecular pathways leading to cellular stress. The heat shock stress pathway (Groh *et al.*, 1996) has previously been reported to regulate MICA and MICB expression. It was also reported that the DNA damage response in human cell lines induced the expression of ULBP1, 2, and 3 and in some studies MICA and MICB (Gasser *et al.*, 2005; Soriani *et al.*, 2009). In terms of infection, HCMV upregulates MICA (but only the truncated MICA*008 molecule), MICB and ULBPs expression on infected cells (Groh *et al.*, 2001)

NKG2D engagement induces NK cell degranulation and while some data suggests that the soluble ligands stimulate cytokine secretion (Andre *et al.*, 2004; Lanier, 2008) others have reported they inhibit NK cell functions (Waldhauer and Steinle, 2006; Waldhauer *et al.*, 2008). NK cell activation via NKG2D is triggered by the tyrosine phosphorylation of the adaptor proteins, which induces a cascade of events. As mentioned, NKG2D signals through the adaptor molecule DAP10, which has a small cytoplasmic domain containing only one known signalling motif (YINM). DAP10 phosphorylation recruits either PI3K through the p85 subunit (Wu *et al.*, 1999) or the adaptor Grb2 (Chang *et al.*, 1999).

1.5.1.3 Other receptors

Some other activating receptors do not contain ITAMs or associate with adaptor molecules carrying ITAMs. Among these, CD2, 2B4, DNAM-1 and NKp80 are included and some of these receptors act as co-stimulators (Bryceson *et al.*, 2006b).

NKp80

NKp80 was identified in 2001 during the investigation of novel NK cell receptors able to trigger NK cell cytotoxicity (Vitale *et al.*, 2001). NKp80 is expressed on all NK cells and a subset of CD8 T cells and γδ T cells. Welte *et al*. demonstrated that it recognises and binds to the activation-induced C-type lectin (AICL) receptor (expressed on myeloid cells), stimulating NK cell cytotoxicity and cytokine secretion (Welte *et al.*, 2006). The transmembrane region of NKp80 does not contain classic ITAM-adaptors; hence the signalling transduction pathway remained unknown until very recently. Using the cell line NK-92MI (an IL-2 dependent cell line), it was reported that NKp80 signals through an atypic hemi-ITAM activating Syk-kinase pathway (Dennehy *et al.*, 2011).

DNAM-1

DNAM-1 (CD226) is a receptor member of the Ig superfamily, expressed not only by NK cells but also by platelets, monocytes, T cells and some B cells (Lanier, 2005). DNAM recognises CD155 and CD112 receptors, both of them are upregulated in tumour cells (Bottino *et al.*, 2003). The interaction between CD155 and CD122 with DNAM enhances NK cell-mediated cytotoxicity and cytokine secretion (Tahara-Hanaoka *et al.*, 2004). More recently, the interaction between DNAM-1 and CD155 demonstrated the ability of NK cells to mediate suppression of metastases in a mouse model (Chan *et al.*, 2010). The signalling triggered by DNAM-1 activation is not well characterised.

1.5.1.4 Signalling downstream activating receptors

Contrary to T and B cells, NK cells do not express a single activating receptor, instead, a wide variety of receptors are expressed by NK cells, which can synergise to regulate NK cell functions (Bryceson *et al.*, 2006a). NKp30 and NKp46 can associate with CD3ζ and FcεR-γ whereas NKp44 associates with DAP12. These signalling partners contain an ITAM. Briefly, when NCRs are engaged, Src mediates the phosphorylation of the ITAM sequence on the associated molecule that results in the recruitment and binding of tyrosine kinases Syk and ZAP70 to the ITAM. Syk and ZAP70 phosphorylate transmembrane molecules like LAT (linker for activation of T cells) or NTAL (non-T-cell activation linker) leading to phosphorylation of several signalling complexes, including PI3K, phospholipase C (PLC-γ1 and PLC-γ2), and VAV-1, 2 and 3 (figure 1.12).

NKG2D associates with DAP10. The short cytoplasmic tail of DAP10 contains a tyrosine-based signalling motif that can be phosphorylated by Src kinases and bind PI3K or the molecule Grb2. PI3K is essential for the production of PIP_3 (phosphatidyl-inositol-3,4,5-trisphosphate), needed for the recruitment of PLC-γ1, PLC-γ2 and VAV-1. VAV-1 is recruited by Grb2 and plays a key role in the induction of actin reorganisation and polarisation of the microtubule organising centre (MTOC) (Cella *et al.*, 2004) and is also important for the activation of SLP76 and PLC-γ2 (figure 1.11). NKG2D does not involve signalling through ZAP70 or Syk, and does not require transmembrane adapters such as LAT or NTAL (Chiesa *et al.*, 2006).

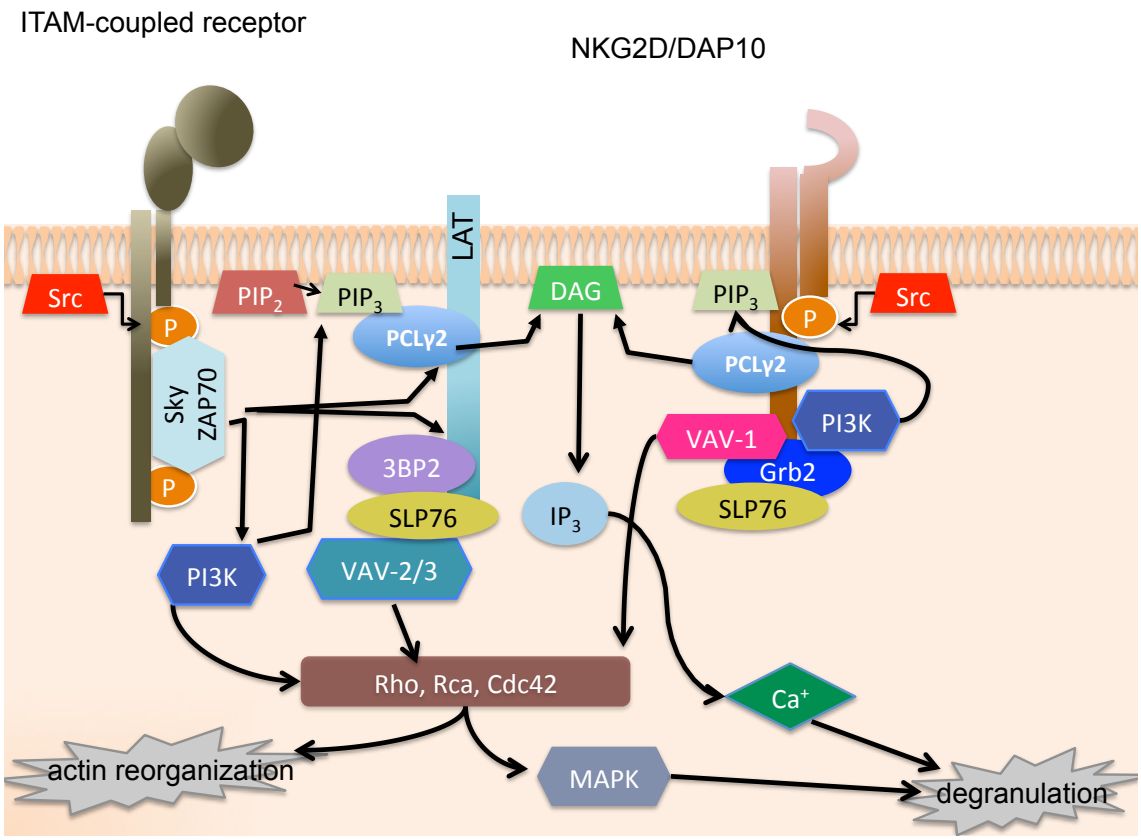


Figure 1.12. Activating pathways. The signalling pathways of activation through ITAM-coupled receptors and DAP10 are shown in the figure. Adapted from Watzl *et al*, 2010 with permission from John Wiley and Sons.

1.5.2 Inhibitory receptors

NK cells have receptors that trigger killing mechanisms, as well as receptors that inhibit these mechanisms in order to protect self-cells. As described previously, the process by which NK cells recognise self from non-self was termed several decades ago by Karre in a theory called “missing self-hypothesis” (Karre *et al.*, 1986). In 1995 the molecular mechanisms of the inhibitory signals were revealed by the identification and cloning of MHC class I binding NK cell receptors (Colonna and Samaridis, 1995; Moretta *et al.*, 1995). Although this theory was proven correct, it is known today that other mechanisms and molecules apart from MHC-ligands inhibit NK cell activity. Some receptors act upon MHC class I molecules, whereas others bind to non-MHC class I ligands. These receptors include the inhibitory KIRs that bind to classical MHC class Ia molecules (HLA-A, -B, and –C) and the inhibitory CD94- natural killer group 2A (NKG2A) heterodimeric C-type lectin-like receptors that bind to the non-classical MHC class Ib (HLA-E) (Lanier, 2001; Lanier, 2008). Even though these receptors differ in their extracellular domains, there is one particular motif that defines their inhibitory function: the ITIM motif. When these receptors engage their ligands, recruitment of

different phosphatases suppresses NK cell responses by dephosphorylating the protein substrates of the tyrosine kinases linked to activating NK cell receptors (Ravetch and Lanier, 2000) including the protein tyrosine phosphatases (PTP) SHP-1 and SHP-2 (Vivier *et al.*, 2004).

Killer Ig-like receptors

KIR receptors belong to the Ig-superfamily and comprise transmembrane glycoproteins with two or three Ig-like extracellular domains (Colonna and Samaridis, 1995). The KIR receptor family may represent the most versatile and polymorphic one among the receptors expressed by NK cells (Yawata *et al.*, 2002). KIRs glycoproteins can be divided into two subgroups based on the number of Ig-like extracellular domains; KIR3D contains three Ig-like domains and KIR2D only two Ig-like domains (Moretta *et al.*, 1996). KIRs may have short or long cytoplasmic tails (S or L) and can transduce activating or inhibitory signals. The long cytoplasmatic tails of KIRs contain an ITIM motif in their cytoplasmic domain responsible for inhibitory signals. Although all KIRs interact with MHC class I molecules, they possess allele specificity. KIR2D recognises primarily HLA-C alleles whilst KIR3D recognises HLA-A3, HLA-A11 and HLA-B (Lanier, 2005). KIRs inhibit NK cell activation in several ways. For instance, the binding of an inhibitory KIR to a MHC class I molecule blocks the phosphorylation of the activating receptors NKG2D and 2B4 (Natarajan *et al.*, 2002) or blocks the accumulation of actin filaments on the immune synapse (Endt *et al.*, 2007). The S forms (KIR2DS and KIR3DS) do not contain ITIM motifs; instead of inhibitory signals they transmit activating signals (Moretta *et al.*, 2001).

Signalling of inhibitory KIRs

During the signalling through ITIM, PTPs (SHP-1 and SHP-2) or the inositol phosphatase SHIP are recruited to the activating motifs of adaptor/receptor molecules (Bakker *et al.*, 2000). The recruitment of SHP-1 and SHP-2 to the ITIM motif blocks the progression of activating signals through dephosphorylation of other molecules such as Syk, PLC γ , VAV-1, transmembrane-associated adaptor proteins (CD3ζ), and cytoskeletal structures (actin and myosin) (Ravetch and Lanier, 2000; Lin *et al.*, 2004). ITIM-containing receptors also bind to additional molecules through their phosphorylated ITIMs, for instance, KLRG1 binds SHIP and KIRs bind to β-arrestin 2, facilitating SHP-1 and SHP-2 recruitment to the inhibitory KIR (Yu *et al.*, 2008). SHIP dephosphorylates PIP_2, a PLCγ substrate, preventing PKC activation and calcium signalling (Ravetch and Lanier, 2000). Studies have shown that ITIMs from KIR

preferentially recruit SHP-1 (Ono *et al.*, 1997) known to dephosphorylate VAV-1, which has a central role in Rac-1-dependent actin cytoskeletal and receptor arrangement during the synapse formation (figure 1.13A) (Watzl and Long, 2003; Endt *et al.*, 2007). A second component of the inhibitory signalling by KIR, and also by CD94/NKG2A, involves the small adaptor molecule Crk, which becomes phosphorylated and binds to Abl (Peterson and Long, 2008). The Abl-Crk binding disrupts the complex Cbl-Crk-C3G, known to contribute to NK cell activation through Rap1 activation (figure 1.13B).

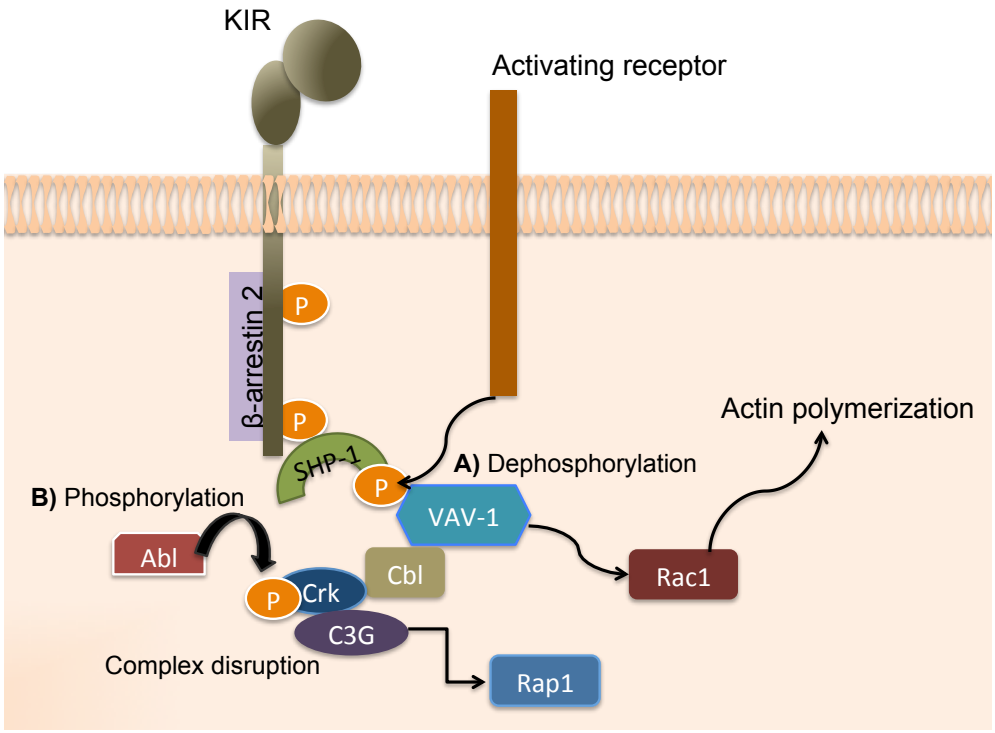


Figure 1.13. ITIM signalling in KIRs. SHP-1 and SHP-2 recruitment to phosphorylated ITIMs is enhanced by the binding of β-arrestin 2. A) Activating receptors usually phosphorylate Vav1, however, SHP-1 dephosphorylates Vav1 and inhibits Rac1 activation preventing actin polymerization. B) Upon engagement of inhibitory receptors, Abl induces the phosphorylation of Crk, resulting in the disruption of the complex Cbl-Crk-C3G. Modified from Watzl and Long, 2010 with permission from John Wiley and Sons.

CD94/NKG2

As mentioned earlier, NKG2 family members couple with CD94 and form heterodimers. NKG2C and NKG2E transmit activating signals while NKG2A transduces inhibitory signals. Unlike KIRs, CD94/NKG2A recognises only one molecule, the non-classical and MHC class I molecule HLA-E. Since HLA-E expression relies on the presence of bound MHC class I leader peptides, CD94/NKG2A serves as an indirect pathway of monitoring HLA class I molecules (Braud *et al.*, 1998; Kaiser *et al.*, 2005). HLA-E assembles at the ER using the leader peptides from HLA-A, B, C and G (Braud *et al.*, 1997). If MHC class I downregulation occurs, HLA-E expression is reduced, rendering

CD94/NKG2-HLA-E interaction as a key mechanism used by NK cells to monitor MHC class I molecules (Kaiser *et al.*, 2005).

Others

Leukocyte Ig-like

Another group of inhibitory receptors from the Ig superfamily is the LIRs or the Ig-like transcripts (ILTs). Only two out of the thirteen members of the LIR family genes encode for inhibitory receptors, LIR1 and LIR2, but only one is expressed on NK cells (LIR1). Although LIR1 contains 4 ITIM motifs in its intracellular region, unlike inhibitory KIRs LIR1 binds with low affinity to MHC class I molecules (HLA-A, HLA-B, HLA-C and non-classical HLA-G) (Colonna *et al.*, 1999). However, the most efficient LIR1 binding is observed with HLA-G (Gonen-Gross *et al.*, 2003).

MHC class I-independent inhibition

As predicted by the "missing-self" hypothesis, NK cell inhibition is mainly regulated by MHC class I molecules, but in some instances, their absence does not augment susceptibility to NK cell-mediated killing. In addition to MHC class I, other molecules contribute to the negative regulation of NK cell activity. For example, the carcinoembryonic antigen (CEA)-related molecule I (Markel *et al.*, 2002; Stern *et al.*, 2005), the killer cell lectin-like receptor G1 (KLRG1) (Ito *et al.*, 2006), NK cell receptor protein 1A (NKR-P1A, CD161) (Lanier *et al.*, 1994), inhibitory receptor protein 60 (Cantoni *et al.*, 1999) and sialic-acid-binding immunoglobulin-like lectins (SIGLECs) (Nicoll *et al.*, 1999).

1.5.3 2B4, a receptor with dual function

2B4 is a member of the SLAM-related receptor family expressed on all NK cells and binds to CD48 on other cells; its cytoplasmic tail contains four Ig-like motifs called ITSM (Claus *et al.*, 2008). Upon phosphorylation of the ITSM motif, 2B4 can bind to small SH2-domain containing adapters such as SAP intracellular protein, EAT-2 or the Scr homology 2 domain-containing protein tyrosine phosphatase SHP-1 and SHP-2. SAP can recruit Fyn, which facilitates downstream signalling to modulate effector function (Veillette, 2006). Another adaptor protein able to bind phosphorylated 2B4 is 3BP2, able to interact with VAV-1, LAT and PLC-γ. It has also been shown that 2B4 stimulation leads to phosphorylation of Cbl, Grb2 and SHIP (figure 1.14) (Bottino *et al.*, 2000; Chen *et al.*, 2004).

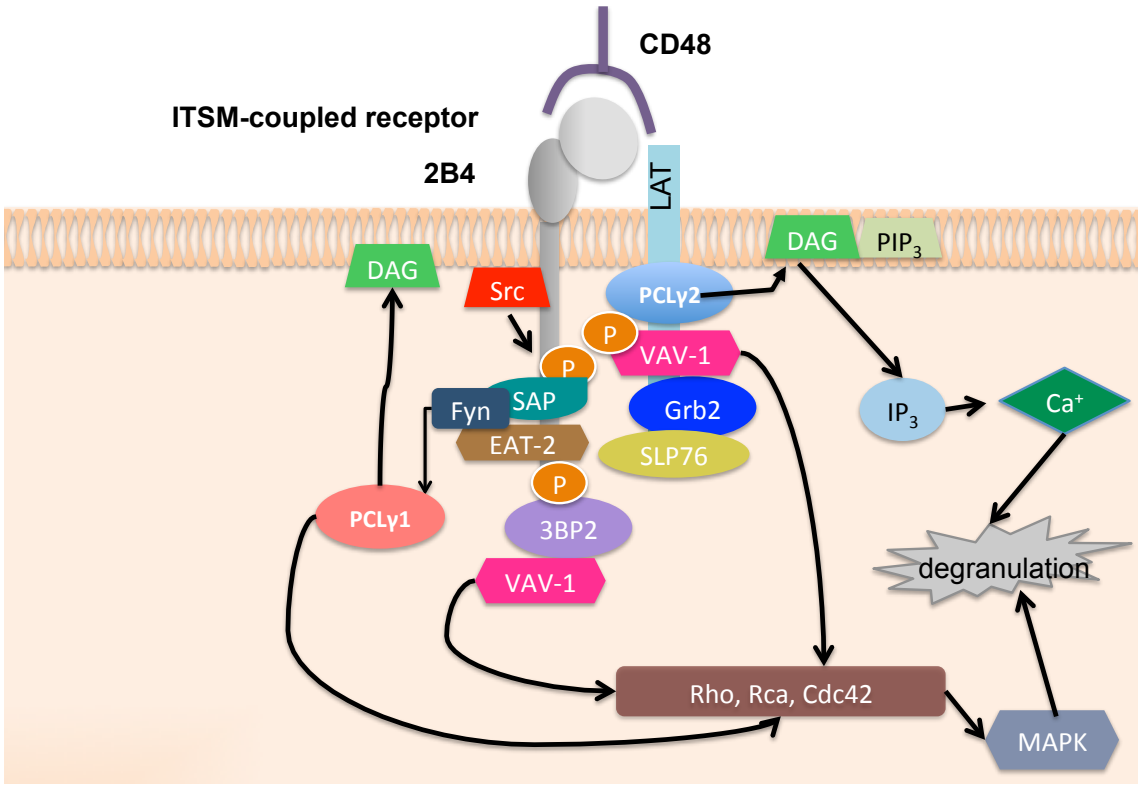


Figure 1.14. 2B4 signalling. 2B4 signalling is initiated upon CD48 binding, followed by phosphorylation of the ITSM sequences by Src kinases. 2B4 associates with LAT and SAP directly, LAT activates PCL-γ and Grb2 whereas SAP binds to Fyn, both molecules promoting NK cell activation. Modified with permission from Elsevier. (McNerney *et al.*, 2005)

2B4 plays a role as activating receptor on mature NK cells, although it is thought to also serve as a co-stimulatory receptor (Bryceson *et al.*, 2005). The main evidence comes from experiments in which NK cell-resistant targets were transfected with the 2B4 ligand CD48, rendering them susceptible to NK cell-mediated killing and triggering IFN-γ production (Tangye *et al.*, 2000). Additionally, an inhibitory role was assigned to 2B4 during the study of NK cell development. It was suggested that this receptor transmits inhibitory signals early on in NK cell development, when the acquisition of activating markers occurs, as a mechanism to prevent self-killing (Sivori *et al.*, 2002). The understanding of this dual nature of 2B4 is not completely understood although a model in which 2B4 is dynamically modulated by the engagement of CD48 and SAP availability has been proposed (figure 1.15) (Chlewicki *et al.*, 2008), further studies will reveal the mechanisms regulating 2B4 signalling.

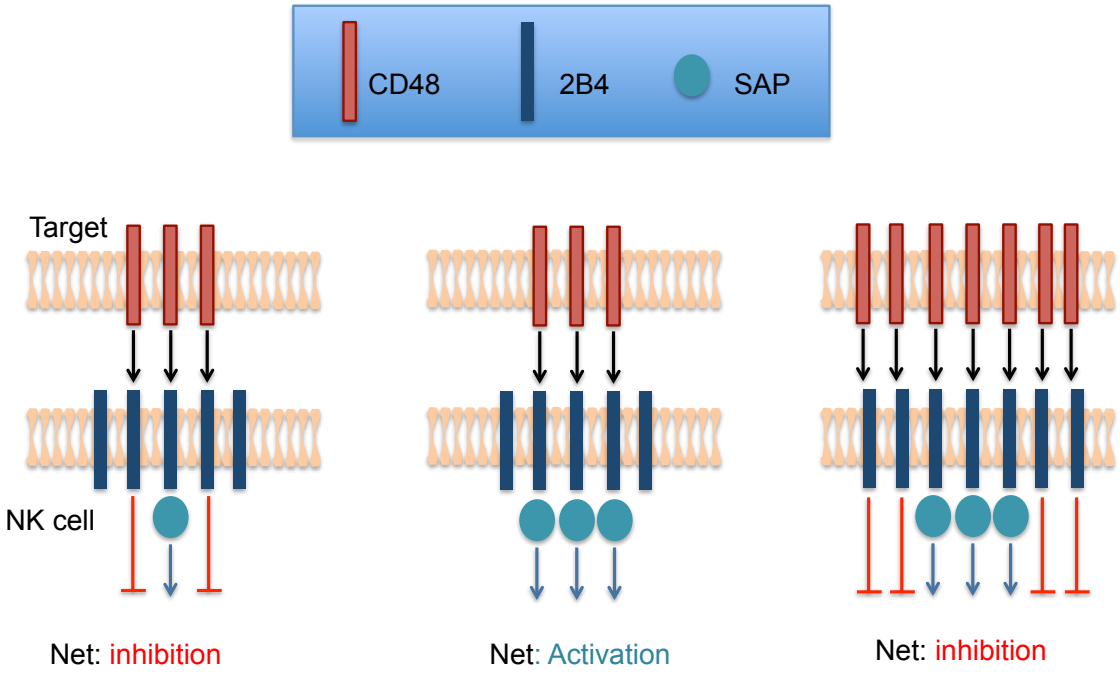


Figure 1.15. Dual function of 2B4 receptor. NK cell activation occurs when the engagement of CD48 with 2B4 in the presence of enough SAP protein (middle panel). Inhibition occurs when not enough SAP protein is available (left panel) or when increased receptor expression makes SAP expression limiting (Waggoner and Kumar, 2012). Reproduced with permission from Frontiers in Immunology under the Creative Commons Attribution 3.0 Unported License.

1.5.4 Adhesion and trafficking Natural Killer cell receptors

Recently, more importance has been given to the receptors affecting adhesion and trafficking of NK cells. NK cell develop in the BM, but they traffic to and from different lymphoid and non-lymphoid tissues. Now it is known that adhesion and trafficking receptors play a key role in NK cell functions in health and disease.

1.5.5 Integrins and selectins

Selectin family

NK cells express L-selectin (CD62L), expressed preferably by the $CD56^{bright}$ NK cell subset. In mice, L-selectin facilitates the adhesion to peripheral LNs and is not needed for recruitment of NK cells to the liver, spleen and lung (Fogler *et al.*, 1996; Chen *et al.*, 2005). Similarly, it has been shown in humans that L-selectin is involved in leukocyte adhesion to peripheral LNs (Uksila *et al.*, 1997). L-selectin expression can be modulated through different stimuli such as IL-2, IL-15 and TGF-β, which downregulate its expression whilst IL-12, IL-19 and IFN-α upregulate it.

Integrins

NK cells express a variety of β1, β2 and β7 integrins. Among β1 integrins α5β1, α4β1, α6β1 serve as receptors for fibronectin, VCAM-1 and laminin respectively (Gismondi *et al.*, 1991). The expression of integrins varies according to the activation state of NK cells; α5β1 and α4β1 expression is acquired during activation whilst α6β1 expression is downregulated (Perez-Villar *et al.*, 1996). The α4 subunit is able to associate with another β chain, β7, giving the α4β7 receptor a different function. NK cells express α4β7, which allows binding to the mucosal vascular addressin MAdCAM-1.

Members of the β2 family allow cell-cell interactions and NK cells express all members from this family (Timonen, 1997). Studies on signalling events after β2 binding on NK cells have shown the rapid phosphorylation and activation of proline-rich tyrosine kinase 2 (Pyk2) (Gismondi *et al.*, 2000). Pyk2 interacts with several signalling or cytoskeletal molecules like Src family members, Grb2 and paxillin (Salgia *et al.*, 1996; Gismondi *et al.*, 1997). A member of the β2 family, LFA-1 (CD11a/CD18), plays a key role in the immunological synapse formation, endothelial binding (Allavena *et al.*, 1991) and functions as a costimulatory receptor on NK cells (Davis *et al.*, 1999). LFA-1 expression is tightly regulated in NK cell subsets, and can be upregulated upon activation with cytokines such as IL-2 or IL-12 (Allavena *et al.*, 1994).

1.5.6 Chemokine Natural Killer cell receptors

The recruitment of leucocytes into different tissues is governed by a superfamily of inflammatory mediators named chemokines. Chemokines play an important role in the regulation of NK cell migration.

NKP and iNK cells can mature in different organs/tissues (Freud *et al.*, 2005). Chemokine receptors and adhesion molecules are expressed differently on NK cell subsets, suggesting different trafficking patterns for these subsets depending on the chemokine receptor expression. G protein-coupled receptors (GPCRs) are membrane proteins that possess seven-transmembrane-domains. Through interaction with GPCRs, chemokines mediate physiological processes including immune system development and pathological processes such as tumorigenesis and cancer metastasis. Chemokines have been divided into four major groups according to the arrangement of cysteine residues in the N-terminal region: CXC, CC, CX3C and C subfamilies. CXC chemokines attract neutrophils and lymphocytes, CC attract mononuclear cells, basophils and eosinophils, CX3C attract T cells, NK cells and monocytes and C chemokines attract T cells. Chemokines are produced either

constitutively or in response to stimuli. Some chemokines bind to more that one receptor and some receptors recognise more than one chemokine.

NK cells express several C, CC, CXC, and CX3C chemokines. The chemokine receptor expression is commonly heterogeneous and depends on the NK cell subset and activation state (Gregoire *et al.*, 2007). Table 1.2 shows the main chemokine receptors expressed by NK cell subsets. NK cells commonly lack the expression of CCR1-7 and CCR9, but the $CD56^{bright}CD16^{-}$ subset can express CCR5 and CCR7, with the latter being mainly involved in homing to LN (Campbell *et al.*, 2001). In terms of CXCR and CX3CR, $CD56^{dim}CD16^{+}$ cells express high levels of CXCR1 and CX3CR1, low levels of CXCR2 and CXCR3 and barely any detectable level of CXCR5. Conversely, $CD56^{bright}CD16^{-}$ express high levels of CXCR1 and CX3CR1 and low levels of CXCR1, CXCR2 and CXCR5. Both subsets express high levels of CXCR4, important for migration to the BM (Campbell *et al.*, 2001). In consequence, these subsets strongly respond to ligand of CXCR4, CXCL12 (Taub *et al.*, 1995). Interestingly, it was reported that CXCR4 was highly expressed in NKP cells and that CXCR4 expression decreases as NK cells mature. NKP cells also express CXCR3 and CCR1 that may participate in the recruitment from the BM to other organs (Bernardini *et al.*, 2008).

Table 1.2. Chemokine receptor expression on the main NK cell subsets.

Chemokine	Chemokine ligands	Expression on $CD56^{dim}CD16^{high}$	Expression on $CD56^{bright}CD16^{low}$
CXCR1	CXCL2,3,5,6,7,8	++	-
CXCR2	CXCL1,2,3,5,6,7,8	+	-
CXCR3	CXCL9,10,11	+	++
CXCR4	CXCL12 (SDF-1)	++	++
CCR1	CCL3,5,7,8,13,14,15,16,23	-	-
CCR2	CCL2,7,8,12,13	-	-
CCR3	CCL5,7,8,11,13,14,15,24,26	-	-
CCR4	CCL17,22	-	-
CCR5	CCL3,4,5,8,11,13,14,20	-	++
CCR6	CCL20	-	-
CCR7	CCL19,21	-	++
CCR9	CCL25	-	-
CX3CR1	CX3CL1	++	+

++ Indicates high levels of expression, + Intermediate levels of expression, - undetectable levels of expression

The expression of chemokine receptors on NK cells can be modulated by cytokines. For example, NK cells exposed to IL-12 for 8-10 days upregulate the expression of CCR1, CCR2, CCR4, CCR5 and CCR8, while IL-15 downregulates the expression of CXCR4 and CX3CR1 on NK cells (Inngjerdingen *et al.*, 2001; Barlic *et al.*, 2003). IL-18 has a different effect on NK cells; it induces the expression of CCR7 selectively on $CD56^{dim}$ NK cells, enhancing their capability to migrate to the LN, but does not affect CCR7 expression on $CD56^{bright}$ NK cells (Mailliard *et al.*, 2005). An interesting mechanism by which lymphocytes can acquire surface molecules from antigen presenting cells and express them on their own surface is termed trogocytosis. Noteworthy is that of the capture of CCR7 by NK cells from allogeneic DC or T cells (Marcenaro *et al.*, 2013). This process was facilitated when NK cells expressed KIR2DS1, allowing NK cell traffic to LNs. An important implication of this process could be observed during cord blood transplantation (CBT), in which NK cells would migrate to LN and destroy patient's T cells and DCs preventing graft versus host disease (GvHD) (Marcenaro *et al.*, 2009). Finally, besides conferring migration properties, chemokines can activate NK cells. CC and CXCL10 chemokines enhance NK cell degranulation and NK cell cytotoxicity by promoting NK cell-target interactions (Maghazachi *et al.*, 1996; Nieto *et al.*, 1998).

1.6 Natural Killer cell killing machinery

1.6.1 Recognition of malignant or foreign cells and immunological synapse

NK cells are able to kill without prior sensitisation and recognise cells as "self" through the engagement of KIR with MHC-class I molecules. As suggested by the "missing-self" hypothesis, NK cells are able to lyse foreign or transformed cells when there is low expression or absence of MHC class I molecules. It has now been recognised that although MHC expression plays a key role in this recognition, the balance between the signalling from activating and inhibitory receptors in the NK cell-target cell engagement will dictate the outcome.

More studies have now shed light into the mechanisms that regulate NK cell effector functions. This process involves several stages. First, the actin cytoskeleton has to be rearranged in order to form an immune synapse (IS) between the target cell and the NK cell. During this step, interactions between LFA-1 and its ligand ICAM-1 are crucial to maintain adhesion and initiate the IS. In addition, integrins not only serve as adhesion molecules but also participate in the signalling before moving to the IS (Davis *et al.*, 1999).

The IS provides the platform in which cell-cell signalling occurs and defines a specific area for accurate cytotoxicity and cytokine targeting (McCann *et al.*, 2003). During the cell-cell interaction, if the target is capable of activating NK cells through their receptor expression or lacks of MHC class I molecules, an irreversible cytolytic response is initiated. Recent data suggests that NK cells remain attached to target cells until a second adjacent target is encountered, forming a ternary complex (Choi and Mitchison, 2013). In this scenario, the first target primes activating signals and the second target accelerates the detachment of the previous target. In the same study, it was revealed that a long delay is observed before the first killing occurs, but subsequent killings took place in a shorter time (Choi and Mitchison, 2013).

Once NK cells are activated, the cytotoxic granules are mobilised towards the IS via the MTOC. It was recently demonstrated that granule convergence toward the IS needs Scr kinases; interestingly, cytokines such as IL-2 are able to induce the lytic granule convergence (James *et al.*, 2013). Finally, fusion of the vesicles containing the granules and release of the lytic contents occurs, namely perforin and granzymes.

1.6.2 Perforin and Granzymes

Perforin was isolated from the cytoplasmic granules of CTL and NK cells and demonstrated to lyse rapidly and unspecifically tumour cells, thus playing a pivotal role in granule-mediated killing. Perforin function depends on Ca^{+2} and is sensitive to the temperature and pH. These are important features since they secure the inadvertent pore formation protecting the NK cell. The cytoplasmic granules maintain an acidic pH, once they are released and encounter high Ca^{+2} concentrations and basic pH in the IS, conformational changes and activation of perforin occurs (Voskoboinik *et al.*, 2005). The pores formed have a diameter from 120-170 Å, enough to allow the diffusion of the granzyme molecules, usually of 50 Å in diameter (Law *et al.*, 2010).

Recent studies have enabled insight of the details of granule-dependent NK cell killing. For instance, a study by Lopez *et al*. was able to demonstrate that membrane permeabilisation of a target cell by perforin can be achieved very fast, in 30 seconds. The repair of the target cell membrane is completed within 80 seconds, but even with the short time of frame in which granzymes have to be delivered, rapid apoptosis was observed in the target cells during this study (Lopez *et al.*, 2013). CD107a is a lysosome-associated membrane protein (LAMP1) transported to the NK cell surface when degranulation occurs. Interestingly, no functional roles were attributed to this

marker until recently; Krzewski *et al.* demonstrated that CD107a facilitates the mobility of the transport vesicles involved in perforin trafficking (Krzewski *et al.*, 2013).

Other granules are needed besides perforin for efficient cell killing by CTL and NK cells. In fact, 90% of the protein mass contained in the cytolytc granules consists of a family of serine proteases named granzymes. There are five granzymes described in humans: A, B, H, K and M (Chowdhury and Lieberman, 2008). Not all of them have been well characterised and studied. We know that granzyme H is a chymase, K is a tryptase, so is A, and that M is a metase (Kam *et al.*, 2000). Granzyme A induces cell death through a caspase-independent pathway that results in nicking of DNA and an increase of reactive oxygen species (ROS) (Martinvalet *et al.*, 2005). Less is known about granzyme K, but a recent report showed that granzyme K dependent-apoptosis is triggered in a caspase-independent manner similar to granzyme A (Guo *et al.*, 2010). Actually, it was shown that $CD56^{bright}$ NK cells could attack autologous T cells using a perforin-dependent mechanism mediated by granzyme A and granzyme K (Jiang *et al.*, 2011). Granzyme B is by far the best-characterised granzyme. Several mechanisms of delivery of granzyme B to target cells have been recently identified. Two models have been proposed and are in constant debate. One of them suggests that granzyme B binds to mannose-6 phosphate (M6P) directly on the target surface; endocytosis of the complex (granzyme B-M6P) and release into the cytoplasm causes apoptosis (Motyka *et al.*, 2000). In the second model, perforin mediates the entrance of granzymes forming de novo pores in the cell membrane of the target. Granzyme B greatly contributes to the NK cell–cell mediated killing through the activation of caspases directly or indirectly through the mitochondria (Lord *et al.*, 2003). Granzyme B activates Bid, a death agonist that initiates a cascade of events leading to the release of mitochondrial mediators such as cytochrome *c.* Cytochrome *c* activates caspases that lead to apoptosis (Waterhouse *et al.*, 2004).

1.6.3 Death-receptor mediated cytotoxicity

NK cell cytotoxicity via the granule-exocytosis is highly efficient. Perforin-deficient mice have impaired cytotoxic functions but alternatives pathways are used to destroy malignant cells. NK cell cytotoxicity can occur via death ligands and receptors (TNF and TNFR, respectively). Fas belongs to the TNF receptor family of molecules containing a conserved intra-cytoplasmic “death domain” that indirectly activates the caspase enzymatic cascade and ultimately apoptotic mechanisms in numerous cell types. FasL is expressed almost exclusively by NK cells and CTL and it can exist as a membrane-bound protein or a soluble ligand (Tanaka *et al.*, 1996). The receptor for

FasL, Fas (CD95) is expressed in various tissues and upon ligation it trimerises and causes the endocytosis of the FasL-Fas complex. The death domains then bind to the protein FADD (Fas-Associated Death Domain), which functions as a molecular bridge to caspase 8, a protease that initiates cell death cascade (Gibson *et al.*, 2000).

TNF-related apoptosis-inducing ligand (TRAIL) is another death receptor expressed by NK cells and CTL. TRAIL can bind to 5 different receptors; three of them act as "decoy" (they do not induce apoptosis), while the binding of the other two (DR4 and DR5) leads to the recruitment of the protein FADD initiating the apoptotic-signalling cascade (Gibson *et al.*, 2000). TRAIL can be upregulated upon activation with cytokines such as IL-2 and IL-15 (Smyth *et al.*, 2005).

1.6.4 Cytokine production

Cytokines released by activated NK cells include GM-CSF, TNF-α and regulatory cytokines such as TGF-β and IFN-γ (Newman and Riley, 2007). The co-engagement of some receptors such as NKG2D, 2B4 or CD16 can stimulate IFN-γ and TNF-α secretion (Fauriat *et al.*, 2010). Together with secreted chemokines, these cytokines stimulate inflammatory responses. Cytokines can modulate monocyte, dendritic cell, and granulocyte growth and differentiation, and can also influence acquired immune responses. IL-12 is produced very early during infection and is responsible for driving IFN-γ production in NK cells (Byrne *et al.*, 2004). IFN-γ affects host responses to tumours by restricting angiogenesis and inhibiting viral replication (Yokoyama *et al.*, 2004). Although the secretion of cytokines including IFN-γ has been associated with the $CD56^{bright}$ NK cell subset, new evidence reveals that $CD56^{dim}$ NK cells can also secrete vast amounts of IFN-γ usually within 4 h of cell activation (Fauriat *et al.*, 2010; De Maria *et al.*, 2011).

1.7 Cancer immunotherapy

Immunotherapy treatment involves strategies to enhance the patient's immune system to fight tumour cells. The methods for immunotherapy include the induction of an immune response against cancer using IFNs or other cytokines or cells able to kill malignant cells (cell-based immunotherapy or cellular immunotherapy). During cellular immunotherapy, the patient is administered cells that stimulate anti-tumour activity or that have intrinsic anti-tumour activity (either autologous or allogeneic), such as NK cells, T cells, or CTLs (Armstrong *et al.*, 2001). In addition, allogeneic HSCT is a form

of immunotherapy, as the donor's immune cells will often attack malignant cells in the phenomenon known as graft versus leukaemia (GvL).

1.7.1 Haematopoietic stem cell transplantation

Replacement of the haematopoietic system was a procedure used for the first time in man more than 50 years ago. HSCT is currently used to treat a variety of diseases, such as haematological and autoimmune disorders, neuroblastoma, ovarian cancer, several types of anaemia (aplastic, Franconi's and others) among other diseases (Copelan, 2006).

HSCs can be derived from autologous or allogeneic sources. Autologous HSCs can be extracted from the patient prior to cytoreductive therapy and infused back. Since no histocompatibility barriers exist in this scenario, better engraftment, faster reconstitution but higher rate of cancer relapse are usually observed. Allogeneic HSC are derived from a related or unrelated HLA-matched donor and transplanted into the patient. Treatment of leukaemia by transplantation of BM or PBSC is limited by the scarcity of HLA-matched related or unrelated donor; only 50–60% of patients are eligible. In this scenario, donor cells can attack tumour host cells (GvL) but also normal host cells (GvHD), a major cause of morbidity (Welniak *et al.*, 2007). HLA-matched sibling donors are the best option for allogeneic HSCT avoiding GvHD by HLA matching but maintaining GvL due to minor histocompatibility antigen differences. In the absence of an HLA-matched donor, partial-HLA or unrelated-HLA matches might be necessary.

Successful HSCT can be hindered by graft rejection (lack of initial donor engraftment), infections and malignant disease recurrence (relapse), but one of the major and most important immunological complications is GvHD. GvHD is a multifactorial disease in which cytokine release is enhanced, APC presentation of alloantigens to incoming donor T cells is augmented and an immunological storm is initiated (Przepiorka *et al.*, 1995). Infused donor T cells are able to recognise alloantigens in the recipient, resulting in T cell activation, expansion and proliferation. T cell activation triggers cytolytic and cytokine apoptotic injuries targeting the skin, gut and liver (Ferrara and Reddy, 2006; Ferrara *et al.*, 2009). GvHD therapy includes the suppression of T cell activity using pharmacological immunosuppressive drugs and the suppression of cytokine release to establish donor-host immunotolerance. Additionally, the incidence of GvHD can be reduced by T cell depletion of the graft before transplantation. However, T cell depletion provides a higher risk of opportunistic infections such as

Epstein-Barr virus (EBV), lymphoproliferative disease and relapse rate (Wagner *et al.*, 2005).

Allogeneic HSCT relies on the suppression or elimination of the host immune system, which can prevent engraftment. This can be performed using a conditioning regime, generally radiation or chemotherapy that can be either myeloablative or reduced intensity. Reduced intensity regimes are associated with lower rates of early infections compared to myeloablative regimes, however the risk of late infections are similar. The timing of immune reconstitution determines the timing of infections. The immune reconstitution will depend on the ability of the graft to produce *de novo* lymphoid and myeloid cells in addition to the function of the mature cells contained in the graft. The sequence of immune reconstitution and infections can be divided in three phases after HSCT following myeloablative-conditioning regimes: 1) the first is the aplastic phase, the patient is at risk of fungal, viral and bacterial infections (Ninin *et al.*, 2001), 2) the second phase comprehends the period from the initial HSCs engraftment to the third or fourth month and HCMV is the main infection during this phase due to reactivation 3) the third phase begins after the fourth month, recipients of allogeneic HSCT are susceptible to bacterial infections such as *S. pneumoniae* and *H. influenza*.

Several sources of HSCs have been used during the past years including BM, mobilised peripheral blood and CB (Welniak *et al.*, 2007). A brief description of these sources is presented in the following sections.

1.7.1.1 Bone marrow and mobilised peripheral blood stem cell transplantation

BM is a rich source of HSCs and was the only HSC source for a long time. In 1956, Donnall Thomas performed the first BM transplant (BMT) using infusion from marrow of an identical twin to treat leukaemia (Thomas *et al.*, 1957). Encouraging data was later presented in 1965 showing retention of allogeneic grafts in the treatment of acute leukaemia (Mathe *et al.*, 1965). But BMT was not confined to haematological diseases, in 1968 and 1969 infants with severe combined immunodeficiency disease were transplanted with their HLA-matched siblings (Bach *et al.*, 1968). Now, BMT is used for the treatment of several malignancies including leukaemia, inherited disorders and more recently solid tumours. The method to obtain BM is relatively simple, however sometimes painful for the donor. BM aspirations are performed under general anaesthesia, and later transfused to the patient.

It has been more than 25 years since peripheral blood stem cells (PBSC) were considered for transplants. In the 1960's the term "blood stem cell" was introduced, but it was not until 1981 when the technology allowed the recollection and cryopreservation of enough autologous stem cell numbers that the first blood-stem cell infusion was performed (Goldman *et al.*, 1981). However, compared to the well-established BMT, PBSC numbers retrieved were very low. Several strategies were implemented, such as the temporary expansion of PBSC using haematopoietic growth factors or CXCR4 antagonists. Two candidates to obtain higher PBSCs numbers were G-CSF and GM-CSF. Today, G-CSF is considered the gold standard drug to mobilise PBSC offering advantages over GM-CSF as demonstrated in follow-up studies (Lane *et al.*, 1995). In 2001, a multicentre clinical trial was performed to assess PBSC compared to BM HSC in transplantation. Interestingly, the conclusions of this study revealed that PBSC transplantation had a faster platelet and neutrophil recovery, higher survival and disease-free survival probability (2 years) and no difference in the cumulative incidence of GvHD (Bensinger *et al.*, 2001).

1.7.1.2 Cord blood stem cell transplantation

Since 1939, CB was already a prospect for therapeutic uses, a concept that outside the neonatology was slowly accepted (Halbrecht, 1939). After BMT in the 50's was successfully achieved, awareness of the use of CB grew slowly and it was not until 1989 that the first successful CBT was performed (Gluckman *et al.*, 1989). The use of a source of HSCs that was usually discarded was exciting news that led to CB being considered for the treatment of malignant haematological disorders. There is considerable evidence that placental blood obtained from CB at delivery is a promising HSCs source. The establishment of international CB banks, advances in supportive care and donor graft selection as well as novel clinical approaches aiming at improving engraftment have led to a dramatic increase in the number of CBT performed worldwide (Tse *et al.*, 2008). To date, more than 20,000 unrelated CB transplants have been performed and more than 570,000 units have been collected and stored in more than 50 public worldwide CB banks (http://www.bmdw.org/).

Cord blood transplantation; advantages and disadvantages

HSCT is a potential cure for haematological malignant diseases. However, its application relies on the presence of a suitable HSC donor. Unfortunately, many patients do not have family with a suitable HLA match. CBT is an emerging therapy for patients without matched donors that offer advantages over BM or PB HSCT such as

non-invasive procurement and rapid availability without the lengthy process of screening (Wall and Chan, 2008). Moreover, international studies strongly suggested that CBT has the advantage of greater tolerance for HLA disparity, lower risk of transmitting latent virus infections (HCMV, Epstein-Barr virus), lower incidence of GvHD and identical GvL effect compared to unrelated BM transplantation (Rocha *et al.*, 2004). The lower GvHD rates have been associated with the naivety of CB cells, characterised by the lower production of inflammatory cytokines such as IFN-γ and TNF-α (Chalmers *et al.*, 1998).

Nevertheless, CBT has been limited by numerous disadvantages, for instance the limited number of HSC and a higher risk of infections due to the delayed immune reconstitution. One of the main causes of morbidity after HSCT is the HCMV infection. A recent report suggested that regardless of the early T cell priming after double CBT (day 42), T cells fail to achieve enough numbers to control infection (McGoldrick *et al.*, 2013). Alternatively, omission of T cell depletion showed a unique thymic-independent T cell reconstitution in children that underwent CBT. Although infections were frequent, these were resolved and the incidence of chronic GvHD was low (Chiesa *et al.*, 2012).

To overcome the low doses of HSCs in CB for adult patients, the use of two CB units preceded by a reduced intensity regimen, facilitated engraftment (Barker *et al.*, 2005). It seems that double CBT results in better rates of engraftment compared to single CBT and does not increase GvHD (Barker *et al.*, 2005; Scaradavou *et al.*, 2013). Along the same lines, the co-infusion of a CB and haploidentical peripheral blood $CD34^+$ cells results in a shortened time of neutropenia following CBT, providing some immune protection until the CB $CD34^+$ cells engraft (Fernandez *et al.*, 2003). Another approach to increase HSC doses is the expansion *ex-vivo* of cord blood stem cells (CBSC), this can be performed using cytokines or co-culture with stromal cells that provide the HSCs microenvironment or extrinsic regulators of stem cell fate (Notch) (Robinson *et al.*, 2002; Delaney *et al.*, 2010; de Lima *et al.*, 2012). Other method to deliver high numbers of HSCs is the intra-bone injection of CB cells, which was associated with better engraftment, early platelet recovery and low incidence of GvHD (Frassoni *et al.*, 2010). Finally, an important disadvantage is the inability to collect additional cells from the CB donor in case of relapse or graft failure to perform a donor lymphocyte infusion.

Although higher transplantation-related mortality was observed in CBT, relapse rates and GvHD were lower compared to transplants using PBSC and BM (V. Rocha and E. Baudoux, 2011). The results of the study performed by the Eurocord and the Centre for International Blood and Marrow Transplant Research (CIBMTR) concluded that CBT is

feasible in adults if the CB unit contains high numbers of HSCs and should be considered when no sufficiently HLA-matched donor exists (V. Rocha and E. Baudoux, 2011).

1.7.2 Natural Killer cells in haematopoietic stem cell transplantation

NK cells can have both, a positive and a negative effect on HSCT according to whether they come from the donor or the host. Donor NK cells can potentially enhance engraftment providing GvL and suppressing GvHD. This is the case in particular when there is a KIR ligand-mismatch in the donor to host direction. On the contrary, host NK cells can increase GvHD and reject the graft by lysis of donor HSCs (Farag *et al.*, 2002; Ruggeri *et al.*, 2002; Barao and Murphy, 2003). Pioneering results of Ruggeri et al. (Ruggeri *et al.*, 2002) have reported that allogeneic NK cells can mediate anti-leukaemic effects against AML (acute myeloid leukaemia) cells after haplo-identical HSCT with KIR-ligand incompatibility (Ruggeri *et al.*, 2006). However, the beneficial effects are controversial, especially in advanced haematological diseases (Schaffer *et al.*, 2004; Aversa *et al.*, 2005). The discrepancies among studies could be due to different transplantation protocols, which vary in type of pre-conditioning, degree of T cell depletion, dose of HSCs and treatment post-transplantation. Some studies suggested that NK cells generated after haplo-identical HSCT are blocked at an immature stage and characterised by specific phenotypic features and impaired functions that may negatively affect transplantation outcome (Nguyen *et al.*, 2005).

Intense immunosuppression after CBT along with the naivety of the infused T cells and slow T cell reconstitution generated a long period of immunodeficiency increasing mortality due to infections. Surprisingly, no increased mortality due to relapse has been observed after CBT and studies have shown that the GvL effect is preserved. Studies on hematopoietic reconstitution after CBT showed that NK cells are the first lineage to be reconstituted and comprise most of the lymphocytes in circulation during the first year after CBT. Reconstituted NK cells are able to kill leukaemia cells *ex-vivo* (Beziat *et al.*, 2009) supporting the hypothesis that NK cells may be responsible of the GvL effect observed after CBT and excludes the possibility of T cells performing this role since it takes between 6-12 months for T cells to reconstitute in the patient (Komanduri *et al.*, 2007).

1.8 Natural Killer cell therapy

1.8.1 Why Natural Killer cell therapy?

The use of the immune cells as therapy has been widely explored. NK cells are not the only alluring option for this purpose. In fact, the biology and functions of T and B cells have been studied long before NK cells. T cell therapies have some limitations, mainly due to the escape mechanisms of malignant cells (described in section 1.7.4). Additionally, T cells are known to provide GvHD (Coghill *et al.*, 2011). Unlike T cells, NK cells do not invoke GvHD, as non-malignant cells usually do not express enough activating ligands to induce NK cell cytotoxicity.

Another immune cell subset that has been studied for anti-tumour therapies are DCs. DC therapy aims to prime T cells against specific anti-tumour antigens. The first attempts to cure cancer using DCs therapy were performed in patients with non-Hodgkin's lymphoma (Hsu *et al.*, 1996). Through the years, other types of cancers have been added to the list: prostate cancer, myeloma, colorectal cancer and non-small cell lung cancer. However, this therapy is still far from being optimal, as DC therapy has failed to eradicate malignant cells in many cancer patients and manipulation *in vitro* is costly and time consuming.

Although currently T cell and DC based therapies are under study, NK cell immunotherapy is rapidly progressing. New approaches have been studied, from simple manipulation using cytokines to sophisticated NK cell engineering.

1.8.2 Natural Killer cells in cancer

A vast amount of literature-based knowledge has demonstrated the capacity of NK cells to recognise and lyse tumour cells (Wu and Lanier, 2003). Hence, NK cells have been considered an attractive tool for immunotherapy, as they do not require prior antigen priming like T cells. NK cells hold a great alloreactivity potential (Colonna *et al.*, 1993) that could be used in other scenarios besides HSCT. Studies in malignant glioma patients and neuroblastoma demonstrated that NK cell infusions are safe although partially effective (Ishikawa *et al.*, 2004; Tarek *et al.*, 2012). Several types of cancer could potentially benefit from NK cell immunotherapy and current clinical trials

include pancreatic, lung, head/neck, breast and renal cell carcinomas. Table 1.3 shows some studies using either autologous or allogeneic NK cells.

Table 1.3. NK cell adoptive therapy. Modified with permission from Nature Publishing group. (Luevano *et al.*, 2012b)

Reference	Cancer	NK cell therapy
(Escudier *et al.*, 1994)	Metastatic renal cell carcinoma (MRCC)	Autologous
(Ishikawa *et al.*, 2004)	Malignant glioma	
(deMagalhaes-Silverman *et al.*, 2000)	Metastatic breast cancer	
(Lister *et al.*, 1995)	Lymphoma and breast cancer	
(Miller *et al.*, 2005)	AML	Allogeneic
(Koehl *et al.*, 2004)	Acute Lymphoblastic Leukaemia (ALL) or AML	
(Passweg *et al.*, 2004)	AML and Chronic Myeloid Leukaemia (CML)	
(Arai *et al.*, 2008)	Refractory Renal Cell Carcinoma and Melanoma	
(Bachanova *et al.*, 2010)	B cell non-Hodgkin lymphoma (NHL)	
(Curti *et al.*, 2011)	AML	
(Iliopoulou *et al.*, 2010)	Advanced non-small cell lung cancer (NSCLC)	

1.8.3 Tumour escape

Unfortunately, not all tumours are susceptible to NK cell-mediated killing and different mechanisms evolved by tumour cells underlie this resistance. Studies from experimental models and human cancer revealed some of the mechanisms regulating evasion of tumour cells from NK cells (summarised in table 1.4):

a) Loss of MHC class I molecules: cancer cells may alter the expression of MHC class I molecules. These molecules are known to play a pivotal role in the presentation of tumour antigens to T cells and also modulate NK cell function. This loss can occur in two ways: 1) through the loss of MHC class I proteins that will present these antigens to tumour-specific T cells, or 2) through the loss of

antigen processing function within the tumour cell that is needed to produce the peptide and load it onto the MHC class I molecule (TAP1, TAP2, and components of the immunoproteasome) (Dunn *et al.*, 2002).

b) MICA production: Many tumours produce the soluble stress-induced molecule MICA. This molecule binds to NKG2D on the effector cells (NK cells) leading to endocytosis and degradation of the NKG2D molecule, which inhibits NK cell-mediated killing (Groh *et al.*, 2002).

c) Treg cells: These cells are major mediators of peripheral immune tolerance through the regulation of Th1 and Th2 immune responses. Treg cells considerably suppress the proliferation and the function of $CD4^+$ T cells, $CD8^+$ T cells and NK cells by the secretion of immunosuppressive cytokines, such as TGFβ and IL-10 (Beissert *et al.*, 2006).

   a. Suppressive cytokines:

      i. IL-10: is an immunoregulatory cytokine with potent anti-inflammatory and immunosuppressive activities. IL-10 can suppress DC function and skew T cell responses toward a type 2 immune response and is less effective against malignant cells (Aruga *et al.*, 1997).

      ii. TGF-β: a cytokine that mediates the suppression of immune responses as well as strong inhibition of epithelial-cell growth. It leads to inhibition of DC activation as well as direct inhibition of T cell and NK cell proliferation and function (Wrzesinski *et al.*, 2007).

d) FLIP: Fas-FasL binding plays an important role in immune regulation. Its function includes T cell homeostasis, cytotoxic T cell activity and tumour counterattack. Tumours may overexpress FasL to induce apoptosis of the infiltrated lymphocytes, allowing the tumour to escape the immune response. Fas activation induces trimerisation of the receptor and ligand; subsequently binding to FADD occurs which in turns recruits caspase-8, resulting in cell death by apoptosis. This chain of events can be inhibited by FLIP. FLIP expression correlates with resistance against death receptor-induced apoptosis in a variety of B-cell lymphomas and FLIP-transfected tumour cell lines develop more aggressive tumours *in vivo* (Igney and Krammer, 2002).

Table 1.4. Tumour escape strategies.

Strategy	Mechanism
Ignorance	Lack of danger signals
	Lack of tumour antigens in lymphoid organs
	Growth in immune privileged sites
	Lack of adhesion molecules
	Physical barrier by stroma
Impaired antigen presentation	Mutation or downregulation of tumour antigens
Expression of immunosuppressive factors and molecules	Mutation or downregulation of MHC antigens
	Defects in antigen processing (TAP, LMP [large multifunctional protease] deficiency)
	Cytokines (TGF-β, IL-10, VEGF, etc.)
	Prostaglandins
	RCAS1 (receptor binding cancer antigen expressed on SiSo cells)
Tolerance induction	Anergy induction (lack of costimulatory molecules)
	Immune deviation
	Tregs
	T cell deletion
Apoptosis resistance	Expression of anti-apoptotic molecules
	Downregulation and mutation of pro-apoptotic molecules

1.8.4 Modulation of Natural Killer cell activity

There have been some advances in the development of different immunotherapy strategies throughout the years. NK cells, being recently considered for cancer therapy, can be manipulated to enhance their cytotoxic activity. Different approaches can be taken to enhance effector functions such as use of cytokines or antibodies and genetic modification.

1.8.5 Monoclonal antibodies

To mimic a missing-self environment several groups have recently used antibodies directed against KIRs (figure 1.16). The preclinical characterisation of 1-7F9, a monoclonal antibody that blocks KIR2DL1, KIR2DL2 and KIR2DL3 receptors on NK cells, showed enhanced NK cell-mediated lysis of AML blasts expressing HLA-C *in*

vitro and *in vivo* (Romagne *et al.*, 2009). Later concomitant administration of lenalidomide and 1-7F9, now known as IPH2101, was tested in multiple myeloma cells showing promising results leading to a phase II clinical trial (Benson *et al.*, 2011). The company leading this project, Innate Pharma, announced recently a second phase I trial using IPH2101 combined with Anti-PD-1 antibody nivolumab for the treatment of solid tumours. The results of these trials will be of great value for the continuous effort to combat cancer.

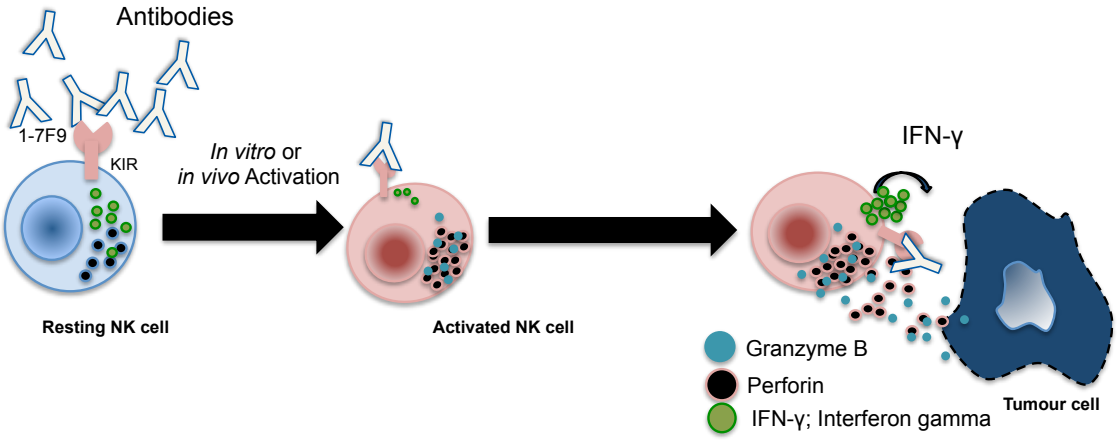


Figure 1.16. Use of monoclonal antibodies to modulate NK cell activity. Infusion of antibodies blocking inhibitory KIRs can be used to activate NK cells and enhance cytotoxicity. Modified with permission from Nature Publishing group. (Luevano *et al.*, 2012b)

1.8.6 Interleukins

NK cell differentiation and activation can be modulated by different interleukins, such as IL-2, -12, -15, -18 and IL-21 (figure 1.17) (Colucci *et al.*, 2003; Becknell and Caligiuri, 2005). IL-2 was the first cytokine used to augment NK cell activity, as experiments *in vitro* revealed increased NK cell cytotoxicity against targets that were previously NK cell resistant (Robinson and Morstyn, 1987; Torelli *et al.*, 2002). Several clinical trials studied the effect of IL-2 administration in patients with cancer (Rosenberg *et al.*, 1987; Rosenberg, 2000). Unfortunately, high doses of IL-2 are toxic and not well tolerated by patients (Fehniger *et al.*, 2002). Nevertheless, the use of low doses of IL-2 was introduced to promote NK cell activity *in vivo*. Although treatment with low doses of IL-2 had undesired effects due to the expansion of the immunoregulatory $CD56^{bright}$ NK cell subset. Further approaches included combination of IL-2 with other factors (Becknell and Caligiuri, 2005) such as the use of IL-2 combined with monoclonal antibodies (Cetuximab for example) that mediate ADCC was able to enhance NK cell activity (Roda *et al.*, 2007). Nevertheless, there has been an inclination for the use of another interleukin that might represent a better option for NK cell activation: IL-15. IL-

15 plays a key role during NK cell development and later in NK cell proliferation and survival; moreover, IL-15 is one of the top twelve agents listed by the National Cancer Institute to be used in immunotherapy (Cheever, 2008). It has been shown that IL-15 is more efficient in expanding NK cells and less cytotoxic than IL-2 (Munger *et al.*, 1995; Ozdemir *et al.*, 2005).

There is a need for more studies investigating the use of cytokines for NK cell activation, either alone or in combination. Studies in mice have demonstrated that NK cells pre-activated with IL-12/15/18 persisted *in vivo* and had a potent anti-tumour activity. In this model, the presence of IL-2 secreted by $CD4^+$ T cells enhanced proliferation and radiotherapy was needed for enhanced effector functions (Ni *et al.*, 2012). It still remains a challenge to translate the encouraging results obtained in mice to humans.

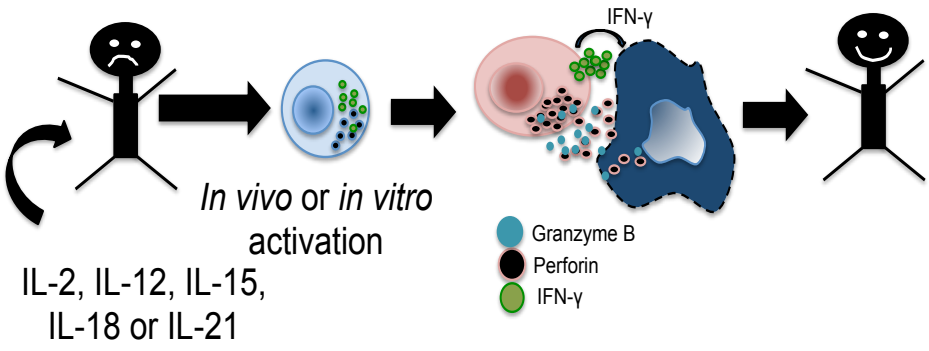


Figure 1.17. NK cell modulation using cytokines. NK cells can be activated with interleukins *in vivo* or *in vitro*, enhancing effector NK cell functions. Modified with permission from Nature Publishing group. (Luevano *et al.*, 2012b)

1.8.7 Natural Killer cell genetic modification

With advancement in technology, new approaches to defeat cancer arise. Within this new technology, the genetic manipulation of cells has opened new hopes for cancer therapy. In this regard, NK cells have been already manipulated *in vitro* in order to enhance proliferation, survival and specific targeting towards malignant cells (figure 1.18). Studies in mice using NK cells bearing a chimeric-antigen specific receptor (against ErbB2 for breast cancer) and in humans directed against CD19 (malignant B cells) have proven an efficient approach against these diseases (Imai *et al.*, 2005; Pegram *et al.*, 2008). Another example is the transduction of the essential NK cell survival cytokine IL-2 into the cell line NK-92 (Nagashima *et al.*, 1998). By transducing IL-2, NK-92 can maintain its own survival and increased the survival of mice bearing

cancer. Similarly, the transduction of IL-15 into NK-92 cells also increases NK cell proliferation and cytotoxicity (Jiang *et al.*, 2008).

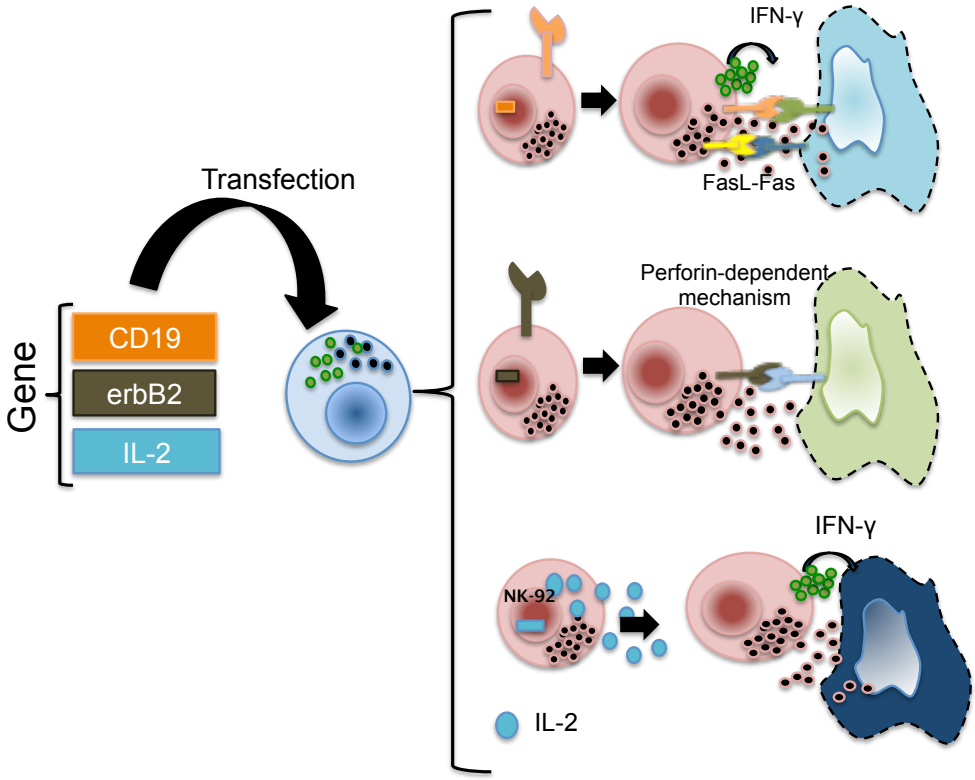


Figure 1.18. Genetic modification of NK cells. Target genes can be transfected into NK cells in order to re-direct NK cell specificity. Likewise, genes that boost NK cell cytotoxicity can also be transfected. Reprinted with permission from Nature Publishing group. (Luevano *et al.*, 2012b)

1.8.8 Adoptive Natural Killer cell immunotherapy

The ability of NK cells to control leukaemia in the HSCT setting led to clinical studies of potential NK cell adoptive immunotherapy. NK cells can be isolated from CB or PB or even produced from HSCs *in vitro* to be used for immunotherapy (figure 1.19). The protocols using autologous NK cells revealed a lack of clinical effects (Burns *et al.*, 2003). Nevertheless, the promising results from HSCT revealing the potential benefits from alloreactive NK cells contributed to the notion of using allogeneic NK cells for adoptive immunotherapy. Miller and colleagues infused allogeneic NK cells together with IL-2 in patients with advanced cancer, demonstrating that donor NK cell infusions were feasible, and 5/19 patients with AML achieved complete remission (CR) (Miller *et al.*, 2005). KIR-mismatch is a requisite for NK cell reactivity; many studies have taken advantage of this scenario and treat different malignancies like AML (Ruggeri *et al.*, 2002) and others (Miller *et al.*, 2005; Pegram *et al.*, 2008) (table 1.3).

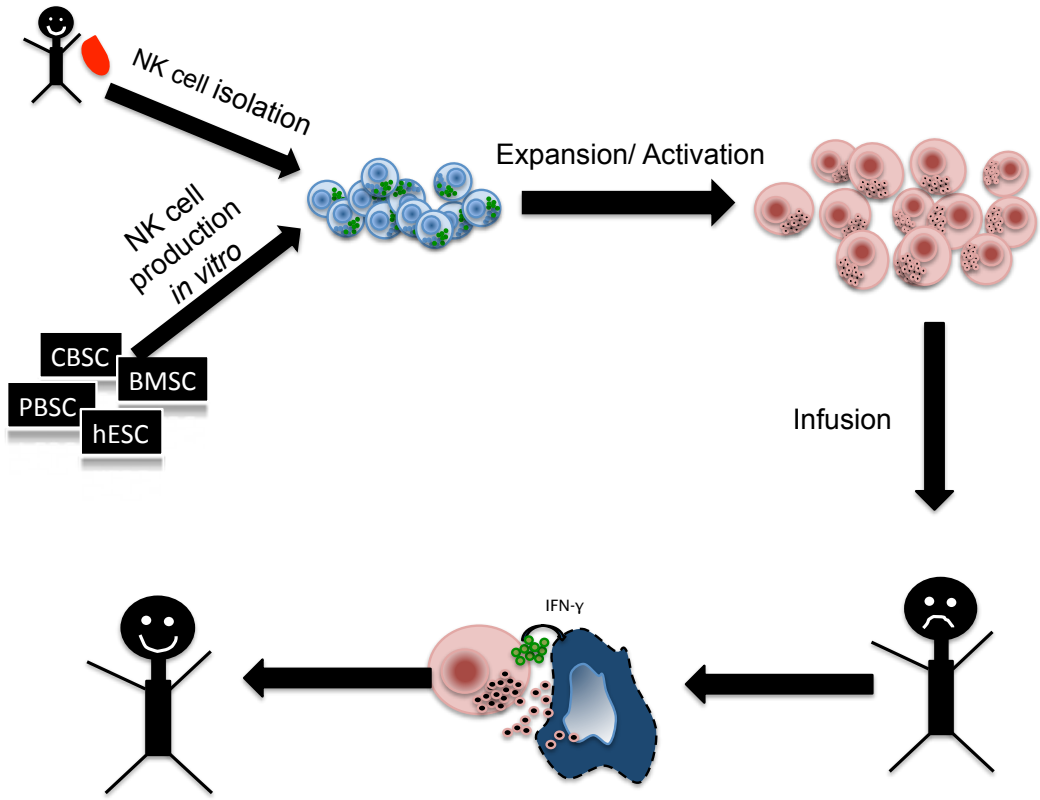


Figure 1.19. NK cell adoptive immunotherapy. NK cells can be directly isolated from healthy donors or produced *in vitro* from HSCs. NK cells can be expanded/activated prior infusion into the recipient. Modified with permission from Nature Publishing group. (Luevano *et al.*, 2012b)

1.8.9 Natural Killer cell generation from haematopoietic stem cells

To date, there have been multiple clinical trials exploring the NK cell potential to eliminate different types of cancer. NK cells have proved to be efficient killers of AML cells, as Ruggeri et al. has elegantly demonstrated *in vitro*, in animal models and finally in humans (Ruggeri *et al.*, 1999; Ruggeri *et al.*, 2002; Ruggeri *et al.*, 2006). There are several sources of NK cells, including PB and CB as well as NK cells generated *in vitro* from HSCs. The following sections address the current approaches on NK cell generation protocols *in vitro*.

1.8.9.1 Types of Natural Killer cell production

The advances of NK cell immunotherapy relies on obtaining high numbers of non-exhausted NK cells that will still be functional and able to proliferate in *vivo*. In this regard, different approaches have been explored. One option is to obtain NK cells directly by isolation from PB or CB although hindered by the limited number of NK cells

that can be obtained and the invasive nature of the procedure in the case of PB. Several protocols assessed the possibility to expand and/or activate PB NK cells *in vitro* using interleukins, cytokines, cell lines or chemicals for further use for immunotherapy (reviewed by Suck et al.) (Suck and Koh, 2010). CB represents a great source of NK cells due to the non-invasive collection procedure, the off-the-shelf availability and less stringent HLA-requirements. NK cells seem to be more naïve, but incubation with interleukins can potentially induce maturation and enhance their functions (Luevano *et al.*, 2012a). Obtaining NK cells from PB and CB has the added advantage of immediate availability regardless of a long-term expansion *in vitro*. Nevertheless, exhaustion due to prolonged exposure to interleukins for NK cell activation is a disadvantage as NK cells are unable to kill and proliferate after infusion into the recipient. It has been reported that 1-2 x 10^7 cells/kg is a safe dose (Passweg *et al.*, 2004) and even doses as high as 1 x 10^8 cells/kg have been tested for treatment of advanced cancers (Lundqvist *et al.*, 2011). Due to the high NK cell doses needed, multiple infusions would be necessary for immunotherapy. Because freezing of NK cells is not considered an optimal procedure due to the reduced killing capacity after thawing (Fujiwara *et al.*, 1986; Voshol *et al.*, 1993), fresh samples are preferred for NK cell therapy in this context. Alternatively, *in vitro* systems to study NK cell ontogeny have shed light on the different factors and cytokines needed in the microenvironment to differentiate HSC into a NK cell (Kobari *et al.*, 2000; McCullar *et al.*, 2008; Bonanno *et al.*, 2009). In light of these findings, a new alternative for obtaining high numbers of NK cells arose; the production of NK cells from HSCs *in vitro*. HSCs can be cryopreserved and used when needed for generation of large numbers of NK cells for immunotherapy. In this context, different sources of HSCs have been used with the direct/indirect aim to generate NK cells *in vitro* including BM (Miller *et al.*, 1994; Mrozek *et al.*, 1996), human embryonic stem cells (hESC) (Woll *et al.*, 2005; Woll *et al.*, 2009), mobilised PBSC (Yoon *et al.*; Zamai *et al.*, 2012) and CBSC (Spanholtz *et al.*, 2010; Spanholtz *et al.*, 2011a; Dezell *et al.*, 2012; Lehmann *et al.*, 2012). All of these HSCs sources have their own advantages and disadvantages; their use will depend mostly on their availability and are briefly discussed in the following sections.

1.8.9.1.1 Human embryonic stem cells

hESC are obtained from 5-7 days old embryos, perhaps making this HSCs source one of the most controversial due to ethical and legal dilemmas. However, the establishment of cell lines from blastocysts has offered new perspectives in the use of hESCs for regenerative purposes and hundreds of cell lines have been established since 1989 (Allegrucci and Young, 2007). There are few protocols using hESC for NK cell generation purposes, for instance Woll et al. used the H9 hESC cell line to produce

NK cells (Woll *et al.*, 2005). In the first publication in 2005, NK cells generated *in vitro* from this cell line expressed activating and inhibitory receptors, including KIRs and CD16. Moreover, NK cells were able to mediate cytotoxicity *in vitro* and produce cytokines. The same group later investigated the potential cytotoxicity of the generated NK cells *in vivo* (Woll *et al.*, 2009). Interestingly, this work included a parallel experiments using CBSC, demonstrating better *in vivo* tumour clearance using hESC-NK cells compared to CBSC-NK cells. Similarly, Knorr *et al*. just recently published a protocol to generate mature and functional NK cells from hESC and induced pluripotent stem cells (iPSC) (Knorr *et al.*, 2013). Currently, immunotherapy using hESC is limited by the potential induction of a severe allogeneic immune response. However, as some authors suggest, a wide genetic diversity could be achievable by expansion of the hESC banks (Woll *et al.*, 2005; Woll *et al.*, 2009).

1.8.9.1.2 Mobilised peripheral blood and bone marrow stem cells

BM harbours a variety of stem cell populations, including endothelial stem cells, mesenchymal stem cells, multipotential adult progenitor cells, pluripotent stem cells and HSCs (Ratajczak *et al.*, 2004). As NK cell development occurs mainly in the BM, certainly HSCs from this source should be able to successfully generate NK cells *in vitro* under the right conditions. Although BM is an alluring source of HSCs, its low numbers of cells along with the invasive collection procedure has disadvantages. Studies using BMSC have mainly focused on the study of NK cell ontogeny. For instance, the first NK cell progenitors ($CD34^+CD7^+$) were described using BM progenitors cultured over a murine feeder layer (Miller *et al.*, 1994). Similarly, Shibuya et al. used $CD34^+$ cells from BM to define the cytokine requirements for NK cell development. Although this study did not include a stromal feeder layer, it was reported that it is in fact needed for optimal NK cell differentiation, as NK cell percentages obtained were very low (Shibuya *et al.*, 1995).

The revolutionary introduction of G-CSF to mobilise HSC became a tool for simplifying and increasing harvests of these cells (Petit *et al.*, 2002). Few studies have performed NK cell generation from PBSC. An interesting NK cell subset was generated during the culture of PBSC, termed NK immunoregulatory (NK-ireg) (Giuliani *et al.*, 2008). This non-cytolytic subset showed a particular phenotype expressing HLA-G, NKp44 and CCR7. Of interest, NK-ireg generation is strictly dependent on trans-presentation of IL-15. Another study using PBSC showed the generation of $CD56^{dim}$ and $CD56^{bright}$ NK cells, the former presenting predisposition to apoptosis (Zamai *et al.*, 2012). Lastly, Yoon *et al*. used PBSC cells from HLA-mismatched donors to generate NK cells *in vitro*

and performed post-transplant NK cell infusions. The generated NK cells had KIRs and NKG2D expression along with cytokine secretion. Overall, no cytotoxicity was reported although GvHD was observed in some cases (Yoon *et al.*).

1.8.9.1.3 Cord blood stem cells

Although controversial, the use of CBSC to generate NK cells *in vitro* has progressed rapidly especially since 2010. Some reports have demonstrated in the past that NK cells generated from CB are immature and less functional compared to the use of other sources such as BM or hESC (Kalberer *et al.*, 2003; Woll *et al.*, 2009). Nonetheless, supporting evidence from Grzywacs and colleagues suggest that CBSC under a proper microenvironment can give rise to functional and mature NK cells (Grzywacz *et al.*, 2006). These studies have also shed light into NK cell ontogeny, proposing the development of NK cells from myeloid precursors (Grzywacz *et al.*, 2011). Similar to Miller *et al*. (Miller *et al.*, 1994), the group of Haddad studied NK cell precursors but in CB finding a lymphoid T/NK precursor ($CD34^{+}CD7^{+}$) able to generate NK cells *in vitro* (Haddad *et al.*, 2004). Even though these protocols were developed to study NK cell development, they provide the fundamentals needed to further development of NK cell protocols. Some other studies using CBSC to generate NK cells are shown in table 1.5.

Table 1.5. NK cells derived from CBSC. Reproduced with permission from Nature Publishing group. (Luevano *et al.*, 2012b)

Reference	Source	Feeder layer	Fold expansion or cell number	Phenotype	Functionality	Purity (end of culture)
(Kao *et al.*, 2007)	$CD133^+$	None	16 x 10^6 cells	$CD56^+$ cells: Perforin(-) and FasL(low)	IFN-γ production (2.77 ng/mL) after IL-12/18 stimulation Cr^{51} assay: 27% killing of K562 at 5:1 E:T ratio	54%
(McCullar *et al.*, 2008)	$CD34^+$	EL08-1D2 and AFT024	123,852 cells	Not described	Not described	Not described
(Haddad *et al.*, 2004)	$CD34^+$	MS-5 stromal cells	112-130 fold	Not described	Not described	95%
(Grzywacz *et al.*, 2006)	$CD34^+$	EL08-1D2	2852 fold	$CD56+CD117^{low}$: NKp44(+), CD161(+), NKp30(+), NKG2A(+/-), NKG2D(+/-) CD94 (+/-), CD16(-) and KIR(-)	Cr^{51} assay: 48% killing of K562 at 5:1 E:T ratio	99%
(Perez *et al.*, 2006a)	$CD34^+$	None	910 x 10^6 cells.	CD161(+), NKG2D(+), NKp46(+), Perforin(+), CD16(+/-), CD94(+/-) and KIR(-)	Cr^{51} assay: 78% killing of K562 at 5:1 E:T ratio	Not described
(Frias *et al.*, 2008)	$CD34^+$	Human BM stromal cells	2 fold	CD16(+/-)	Cr^{51} assay: 15% killing of K562 at 5:1 E:T ratio	25.80%
(Kobari *et al.*, 2000)	$CD34^+$	MS-5 stromal cells	7-27 fold	CD2(+/-), CD7(+/-), CD8(+/-) and CD16(-)	Not described	72% ±7%
(Bonanno *et al.*, 2009)	$CD34^+$	None	Using IL-15 + IL-21: 400 fold	UCB $CD34^+$ derived: KIR(-)CD161(-)CD244(+) UCB $CD34^-lin^-$ derived: KIR(-)CD244(+)CD161(+)	$CD34^-lin^-$ derived NK cells: CD107a assay K562: 59.5% ratio 10:1 (IL-12+IL-15 culture)	92%

E:T; effector to target ratio

1.8.10 Feeder layers used in Natural Killer cell cultures

During adult life, HSCs are maintained in the support microenvironment of the BM. The complete array of blood cell types are generated from HSCs as they differentiate and commit to specific cell lineages. Both, cell differentiation and commitment are controlled by complex interactions within the stromal microenvironment, consisting of different cell types (Lord *et al.*, 1975). In an effort to examine the interactions between HSCs and stromal cells, several *in vitro* cultures using adherent cells from BM have been established (Gartner and Kaplan, 1980). Oostendorp and colleagues undertook the challenge of characterising the haematopoietic microenvironment using mouse embryos (Oostendorp *et al.*, 2002). Studying the different organs that maintain haematopoiesis during embryogenesis, different feeder layers were established according to developmental stage from which they were derived. Haematopoiesis starts in the aorta-gonads-mesonephros region and later takes place in the foetal liver, which becomes a major haematopoietic organ during mid-gestation in mice. Notably, the feeder layer AFT024, derived from day 14.5 gestation of mouse foetal liver, has been shown to maintain HSCs activity for long periods of time (Moore *et al.*, 1997). A major breakthrough using the feeder layer AFT024 for NK cell ontogeny came in 2001, where the need of stromal microenvironment and IL-15 was described to be critical for NK cell differentiation and receptor acquisition (Miller and McCullar, 2001). Later, the comparison between the feeder layers AFT024 and EL08.1D2 was performed in 2008, finding a superior capacity from EL08.1D2 to recapitulate the microenvironment needed for optimal NK cell development (McCullar *et al.*, 2008). Another cell line used for NK cell expansion is M2-10B4 (murine BM) (Pierson *et al.*, 1995), later compared to other cell lines (NRK-49F and NIH-3T3) in the search for factors controlling NK cell expansion (Pierson *et al.*, 1996). These series of studies support the notion that of stromal feeder layers provide the necessary growth factors for optimal NK cell development *in vitro*.

1.8.11 Good manufacturing practice procedures

Good manufacturing practices (GMP) protocols have been established to assure quality of the final product. Although GMP guidelines may vary among countries, the basic concepts remain very similar- the final goal is to safeguard the health of the patients. Good progress has been made in the area of *ex-vivo* NK cell expansion in this regard. Several groups have isolated and expanded alloreactive NK cells for the

treatment of AML (Siegler *et al.*, 2010) or other diseases (Sutlu *et al.*, 2010). Additionally, CB NK cell expansion has been performed using low weight heparin and GMP-compliant procedures demonstrating high fold NK cell expansion (Tanaka *et al.*, 2011).

In terms of NK cell production *in vitro*, the use of some reagents constitute a hurdle to comply with GMP. For example, foetal bovine serum (FBS), needed in culture due to the high content of growth factors, is complex in its contents and varies from batch to batch. Some protocols have been developed avoiding the use of FBS and substituting it with lipids, insulin or other ingredients. An example is that performed by Kao *et al*., in which a serum-free protocol was used to generate NK cells ($CD56^{bright}$) from CB $CD133^{+}$ cells (Kao *et al.*, 2007). Along these lines, other serum-free cultures have been performed, where CB $CD34^{-}lin^{-}$ (Bonanno *et al.*, 2009) or $CD34^{-}CD7^{+}$ (Frias *et al.*, 2008) cells have been used to generate NK cells. Additionally, the work published by Spanholtz *et al.* during the last three years has demonstrated the feasibility of generating NK cells using CBSC and complying with GMP-procedures (Spanholtz *et al.*, 2010; Spanholtz *et al.*, 2011a). This may be the only group so far performing a clinical trial phase I using NK cells generated *in vitro* (NTR2818) and focuses on the treatment of AML patients that were not eligible for HSCT. The development of new reagents for clinical use will certainly speed up the incorporation of other NK cell protocols to be used in immunotherapy.

1.8.12 Trafficking of Natural Killer cells after infusion

One of the main questions regarding NK cell adoptive therapy is their potential trafficking. Initial studies by Walzer *et al*. performed in mice showed that NK cells traffic through blood, spleen, lung and liver (Walzer *et al.*, 2007). Furthermore, Brand *et al*. performed infusion of labelled ^{111}In-NK cells activated overnight to renal carcinoma patients. In order to assess circulation of NK cells in PB, they used a nested PCR technique to detect donor HLA-DRB1 alleles. Post-transfusion, all patients showed circulating NK cells by day 3 and after 7 days all donor cells were cleared. When assessing dissemination of NK cells, they found a systemic distribution with preference in liver, spleen and BM. No activity was observed in LNs (Brand *et al.*, 2004).

1.9 Formulation of the problem and aims of this thesis

Obtaining a large number of NK cells is an essential task and the most significant challenge to the development of successful NK cell adoptive transfer protocols. It has been previously reported that there is a dose-dependent effect of NK cells used in immunotherapy (Alici *et al.*, 2007). Moreover, not only high NK cell numbers are needed, NK cell purity and optimal activation state are regarded as critical factors (Berg and Childs, 2010).

As high numbers of NK cells can be generated *in vitro* from HSCs, CBSC and PBSC are promising sources for this approach. PBSC have become a more accessible cell source due to the use of mobilising agents and CBSC have the advantages of non-invasive collection, less stringent HLA matching and off-the-shelf availability (; Mayani, 2011). The use of cryopreserved HSCs would present a convenient option for immunotherapy. However, different studies reported that the expansion of frozen HSC could often be poor (Boissel *et al.*, 2008a) with a decreased cell count and viability (Beshlawy *et al.*, 2009), while others report that frozen CBSC could be used to generate NK cells (Spanholtz *et al.*, 2010) because of their high proliferative and clonogenic capacity (Moezzi *et al.*, 2005). In addition, with the rapid advancement in technology and new protocols supporting NK cell generation *in vitro*, the need for defining which source of HSCs, CBSC or PBSC, fresh or frozen, are better for this approach is critical. Thus, the specific aims of this thesis were:

1) The optimisation of a NK cell generation protocol through the modification and comparison of a published protocol (chapters 3-5)
2) The study of NK cell development and NK cell generation kinetics from CBSC and PBSC cultures (chapter 3)
3) To investigate the phenotypic and functional characteristics of NK cells generated *in vitro* from CBSC and PBSC (chapters 4 and 5)
4) To determine whether cryopreservation of CBSC has an effect on NK cell phenotypic and functional features (chapters 3-5)
5) To investigate the role of the myeloid marker CD33 on the effector functions of NK cells generated *in vitro* (chapter 6)
6) To study of the effects of IL-12 on phenotypic and functional characteristics of NK cells generated *in vitro* (chapter 6)

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Media, kits, buffers and plastic materials

The culture media used for this thesis are listed in table 2.1.

Table 2.1. Cell culture reagents

CULTURE MEDIA	COMPANY	LOCATION
Alpha MEM without L-glutamine	Lonza	Verviers, Belgium
Dulbecco's Modified Eagle Medium (DMEM) high glucose with L-glutamine	Lonza	Verviers, Belgium
Ham's F12	Lonza	Verviers, Belgium
MyeloCult	Stem cell Technologies	Grenoble, France
RPMI 1640	Lonza	Verviers, Belgium

The different types of serum used during this thesis are listed in table 2.2.

Table 2.2. Serum

SERUMS	COMPANY	LOCATION
Bovine serum albumin (BSA)	Sigma	Poole, UK
Foetal bovine serum (FBS) South America origin	Lonza	Verviers, Belgium
Human Serum type AB (AB serum)	Lonza	Verviers, Belgium
Mouse serum	Sigma	Poole, UK

Cell isolation and labelling kits are listed in table 2.3.

Table 2.3. Cell isolation and labelling kits

KIT	COMPANY	LOCATION
CD34 MicroBead kit	Miltenyi Biotec	Bergisch Gladbach, Germany
CliniMACS CD133 Reagent	Miltenyi Biotec	Bergisch Gladbach, Germany
Human Granulocyte Depletion Kit	Stem Cell Technologies	Grenoble, France
NK cell isolation kit	Miltenyi Biotec	Bergisch Gladbach, Germany
PKH67 Fluorescent Cell Linker Kit	Sigma	Poole, UK

Table 2.4 shows all the solutions and reagents used.

Table 2.4. Solutions and reagents

SOLUTIONS & REAGENTS	COMPANY	LOCATION
1450 microbeta Plus liquid Scintillation counter	Perkin Elmer	Cambridgeshire, UK
AC-IETD-CHO (Granzyme B inhibitor)	Life Technologies	Paisley, UK
β-mercaptoethanol (β-ME)	Life Technologies	Paisley, UK
Bovine Gelatine	Sigma	Cambridgeshire, UK
Chromium 51	Perkin Elmer	Cambridgeshire, UK
Dextran 40	Fresenius Kabi	Barcelona, Spain
Dimethylsuphoxide (DMSO)	Sigma	Poole, UK
DNase	Merck KGaA	Darmstadt, Germany
Ethanolamine	Sigma	Cambridgeshire, UK
Ethylenediaminetetraacetic acid (EDTA) 0.5M ultra pure pH 8.0	Life Technologies	Paisley, UK
Ficoll—Paque PLUS	GE Healthcare	Uppsala, Sweden
GolgiStop™	BD Biosciences	Oxford, UK
Glutamax	Life Technologies	Paisley, UK
Hydrocortisone	Sigma	Dorset, UK
Heparin sodium 1000 IU/ml	Sigma	Cambridgeshire, UK
Ionomycin (Iono)	Sigma	Cambridgeshire, UK
Lympholyte	VH BIO LTD.	Gateshead, UK
L-Glutamine 1640	Lonza	Verviers, Belgium
Magnesium chloride	Sigma	Poole, UK
PBS 10X	Lonza	Verviers, Belgium
Penicillin and streptomycin (Pen-Strep)	Lonza	Verviers, Belgium
Perm/fix Buffer	BD Biosciences	Oxford, UK
Phorbol myristate acetate (PMA)	Sigma	Cambridgeshire, UK
BD Pharma lyse	BD Biosciences	Oxford, UK
Sterile water	Baxter	Zurich, Switzerland
Sodium Selenite	Sigma	Cambridgeshire, UK
Trisodium citrate	Sigma	Poole, UK
Triton 100X	VWR International	Leicestershire, UK
Trypan blue (0.4%)	Sigma	Poole, UK
Trypsin (0.25%) with EDTA	Life technologies	Paisley, UK
Tween 20	Sigma	Poole, UK

Table 2.5 contains the buffers used and their composition.

Table 2.5. Buffers

Buffer	Composition
Blocking buffer for labelling	10% mouse serum in 1X PBS
Freezing solution	90% FBS, 10% DMSO
Labelling buffer (FACS), flow cytometry	1X PBS, 10% FBS
Labelling buffer (MACS) for cell isolation	1% BSA, 2 mM EDTA, 1X PBS
Staining buffer for degranulation assay	1X PBS, 2% FBS, 2 mM EDTA
Thawing buffer	Dextran 40, 5% FBS, 0.63% Sodium citrate, 5 mM $MgCl_2$, 1000 IU/ml DNAse.
Transport media	0.05 μM 2-Mercaptoethanol, 0.63% Trisodium citrate.
Washing buffer for ELISA	1X PBS, 0.05% Tween-20

Table 2.6 lists all the plastic materials used during this thesis.

Table 2.6. Plastic material

MATERIAL	COMPANY	LOCATION
1 ml syringe	BD Plastipack	Madrid, Spain
5 ml ploypropylene round bottom tube	BD Biosciences	Erembodegen, Belgium
Blunt fill needle 21G	BD Biosciences	Erembodegen, Belgium
Cell strainer 40 µM nylon	BD Falcon	Erembodegen, Belgium
Cryotube vials (1.8 ml) foot round	Nunc	Roskilde, Denmark
Falcon tubes, (15 and 50 ml)	Sarstedt	Numbrecht, Germany
LS columns	Miltenyi Biotec	Bergisch Gladbach, Germany
Minisart sterile filter	Sartorius Stedim Biotech	Munich Germany
Paraformaldehyde (PFA)	Sigma	Poole UK
Pasteur pipettes	Fisher	Loughborough UK
Serological pipettes, (5, 10 and 25 ml)	Sarstedt	Numbrecht, Germany
Tissue culture dish (92 x 17 mm) Nunclon surface	Nunc	Roskilde, Denmark
Tissue culture flask (25, 75 and 150 cm^2)	Sarstedt	Numbrecht, Germany
U-bottom 96-well plates	Sarstedt	Numbrecht, Germany
V-Bottom 96-well plates	Sarstedt	Numbrecht, Germany

2.1.2 Cell lines

2.1.2.1 K562

The cell line K562 was established from a 53-year-old female patient suffering from CML (Lozzio and Lozzio, 1975). Cells grow in suspension and form clumps. The K562 cell line carries the Philadelphia chromosome, a chromosome translocation that results in the oncogenic BCR-ABL gene fusion. This cell line is sensitive to NK cell-mediated killing due to the absence of MHC class I antigens and the high expression of NKG2D ligands able to activate NK cells (Bae *et al.*, 2012).

2.1.2.2 EL08.1D2

EL08-1D2 was cloned from an embryonic liver at day 11 of development, and it has been proven to support the generation of NK cells from human haematopoietic precursors (Grzywacz *et al.*, 2011). This cell line was kindly provided by Dr. Robert Oostedrop.

2.1.2.3 Raji

Raji is a lymphoblast-like cell line established in 1963 from an 11-year-old Burkitt's lymphoma patient by Pulvertaft (Pulvertaft, 1964). Raji is an NK-cell resistant cell line.

2.1.2.4 P815

P815 is a mastocytoma mouse cell line derived by the treatment of methylcholanthrene of a DBA/2 male mouse (Gajewski *et al.*, 2001). This cell line is FcγR+ and is used for redirected cytotoxicity assays.

2.1.3 Blood

2.1.3.1 Umbilical cord blood

CB samples were obtained after normal full-term delivery from the Anthony Nolan Cord Blood bank Nottingham, UK or from the Programa Concordia Banc de Sangi Teixits, Barcelona, Spain with written informed consent of the mother. Using routine banking procedures, samples were collected into a cord blood donation bag containing a citrate-phosphate-dextrose anticoagulant buffer and processed within 24 h.

2.1.3.2 Peripheral blood

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy volunteers, upon informed consent. Blood was collected into falcon tubes containing heparin or commercial tubes containing EDTA (BD Vacutainer).

2.1.4 Flow cytometry

2.1.4.1 Antibodies

This work aimed to analyse the expression of activating and inhibitory markers as well as chemokine receptors and integrins on the generated NK cells. Table 2.7 includes the clones and fluorochromes for all the antibodies used in this work.

Table 2.7. Antibodies

Antibody	Company	Catalogue number	Clone	Isotype	Description
7AAD	BD Pharmingen	559925	-	-	Apoptosis marker
Anti-CD16	BD Pharmingen	555403	3G8	IgG1, κ	ADCC assay
Anti-CD33	eBiosciences	14-0338-82	WM-53	IgG1, κ	Blocking assay, myeloid marker
CCR5	R&D Systems	45531	45531	IgG2b, κ	Chemokine receptor
CCR6	R&D Systems	53103	53103	IgG2b, κ	Chemokine receptor
CCR7	BD Pharmingen	552176	3D12	IgG2a, κ	Chemokine receptor
CD3-PerCP	BD Pharmingen	347344	SK7	IgG1, κ	T cell marker
CD7-PE	eBiosciences	12-0079-42	124-1D1	IgG1, κ	Lymphoid lineage marker
CD8-FITC	BD Pharmingen	345772	SK1	IgG1, κ	CTL marker
CD10-PE	eBiosciences	12-0106-41	CB-CALLA	IgG2b, κ	Myeloid lineage marker
CD11a (LFA-1)-FITC	BD Pharmingen	555383	HI111	IgG1, κ	Integrin
CD14-FITC	Immunotools	21279143	MEM-15	IgG1, κ	Monocyte marker
CD19-PE	Immunotools	21270194	LT19	IgG1, κ	B cell marker
CD33-PE	BD Pharmingen	555450	WM53	IgG1, κ	Myeloid lineage marker
CD34-APC	BD Pharmingen	555824	581	IgG1, κ	Stem cell marker
CD34-FITC	BD Pharmingen	555821	581	IgG1, κ	Stem cell marker
CD34-PerCP	BD Pharmingen	345803	8G12	IgG1, κ	Stem cell marker
CD38-APC	BD Pharmingen	555462	HIT2	IgG1, κ	Differentiation
CD45-APC	BD Pharmingen	555485	HI30	IgG1, κ	Differentiation
CD45RA-FITC	BD Pharmingen	555488	HI100	IgG2b, κ	Differentiation
CD48-FITC	BD Pharmingen	555759	TÜ145	IgM, κ	Activating receptor

Antibody	Company	Catalogue number	Clone	Isotype	Description
CD49d-PE	eBiosciences	12-0499-41	9F10	IgG1, κ	Integrin
CD56-APC	BD Pharmingen	555518	B159	IgG1, κ	NK cell marker
CD56-PE	BD Pharmingen	555516	B159	IgG1, κ	NK cell marker
CD94-FITC	BD Pharmingen	555888	HP-3D9	IgG1, κ	NK cell precursor marker
CD95-FITC	BD Pharmingen	555673	DX2	IgG1, κ	Death receptor
CD107a-FITC	BD Pharmingen	555800	H4A3	IgG1, κ	Degranulation
CD117-PE	BD Pharmingen	332785	104D2	IgG1, κ	NK cell precursor marker
CD133-PE	Miltenyi	130-090-853	293C3	IgG2b, κ	Stem cell marker
CD158b-FITC	BD Pharmingen	559784	CH-L	IgG2b, κ	Inhibitory receptor
CD161-FITC	BD Pharmingen	556080	DX12	IgG1, κ	Cytotoxicity
CD226 (DNAM-1)-FITC	BD Pharmingen	559788	DX11	IgG1, κ	Adhesion marker
CD244 (2B4)-APC	eBiosciences	16-2449	PP35	IgG1, κ	Activating/inhibitory receptor
CD253 (TRAIL)-PE	BD Pharmingen	550516	RIK-2	IgG1, κ	Death receptor
CD314 (NKG2D)-PE	Miltenyi	130-092-672	BAT221	IgG1, κ	Activating receptor
CXCR1	R&D Systems	FAB330P	42705	IgG2a, κ	Chemokine receptor
CXCR4	R&D Systems	FAB170P	12G5	IgG2a, κ	Chemokine receptor
CXCR7	R&D Systems	FAB42271P	358426	IgG2a, κ	Chemokine receptor
Granzyme B-FITC	BD Pharmingen	560211	GB11	IgG1, κ	Cytotoxicity
IFN-γ-FITC	BD Pharmingen	554551	4S.B3	IgG1, κ	Immunomodulatory
IL-2Rα (CD25)-APC	eBiosciences	17-0259	BC96	IgG1, κ	Interleukin receptor
IL-2Rβ (CD122)-PE	BD Pharmingen	554525	Mik-B3	IgG1, κ	Interleukin receptor
IL-12Rβ1 (CD212)-APC	BD Pharmingen	558708	2.4 E6	IgG1, κ	Interleukin receptor

Antibody	Company	Catalogue number	Clone	Isotype	Description
IL-15Rα -PE	eBiosciences	12-7159-42	eBioJM7A4	IgG2, β	Interleukin receptor
IL-18R-FITC	eBiosciences	11-7183-42	H44	IgG1, κ	Interleukin receptor
Integrin β7-FITC	eBiosciences	11-5867-42	FIB504	IgG2a, κ	Integrin
Isotype control-FITC	BD Pharmingen	554679	MOPC-21	IgG1, κ	IgG1 Control
Isotype-IgG1	BD Pharmingen	554721	107.3	IgG1, κ	ADCC assay, control
NKG2A-PE	Beckman Coulter	IM3291U	Z199	IgG2b, κ	Inhibitory receptor
NKG2C-PE	R&D Systems	FAB138P	134591	IgG1, κ	Activating receptor
NKG2D-PE	Miltenyi	FAB139A	BAT221	IgG1, κ	Activating receptor
NKp30-PE	BD Pharmingen	558407	P30-15	IgG1, κ	Activating receptor
NKp44-APC	Biolegend	325110	P44-8	IgG1, κ	Activating receptor
NKp46-APC	BD Pharmingen	558051	9E2/NKp46	IgG1, κ	Activating receptor
NKp80-PE	Biolegend	346706	5D12	IgG1, κ	Activating receptor
Perforin-PE	BD Pharmingen	556437	δG9	IgG2b, κ	Cytotoxicity

2.1.5 Molecular biology

2.1.5.1 RNA extraction

The RNeasy Mini Kit from Qiagen (Crawley, UK) was used to extract RNA. The cleaning of pipettes and working surfaces was performed with RNaseZap (Pasley, Life Technologies, UK).

2.1.5.2 Complementary DNA

Reagents for the reverse transcription reaction are listed in table 2.8.

Table 2.8. Complementary DNA reagents

REVERSE TRANSCRIPTION REAGENTS	COMPANY	LOCATION
dNTPs	Life Technologies	Paisley, UK
DTT 0.1M	Life Technologies	Paisley, UK
Random primers 500 µg/ml	Promega	Southampton, UK
Recombinant RNA sin Inhibitor	Promega	Southampton, UK
RNAse/DNAse free water	Life Technologies	Paisley, UK
RNase Zap	Life Technologies	Paisley, UK
SuperScript II Reverse Transcriptase	Life Technologies	Paisley, UK

2.1.5.3 Real time PCR

The Precision 2X qPCR (real time PCR) Master Mix with Low ROX and SYBER green (Primer Design, Southampton UK) was used for all real time PCRs reactions. The reference gene kit geNorm (Primer Design) was used to determine optimal housekeeping genes for PB and CB samples. The selected genes include:

- Homo sapiens actin, beta (ACTB), mRNA.
- Homo sapiens ubiquitin C (UBC), mRNA.
- Homo sapiens beta-2-microglobulin (B2M), mRNA.
- Homo sapiens topoisomerase (DNA) I (TOP1), mRNA.
- Homo sapiens ATP synthase, (ATP5B), mRNA.

The reactions were carried out using 96 well optical reaction plates with barcodes and optical adhesive films from Applied Biosystems (Ca, US). The real time PCRs were performed using an AB 7500 Real time PCR system (Applied Biosystems, Ca, USA).

2.1.5.4 Primers

The sequence of the primers used is shown in table 2.9. All the primers were purchased from Sigma.

Table 2.9. Primer sequences

Primer	Sequence	Source
2B4 (CD244)	F- 5'-GGTACAGAGGGAGCAAGCTG -3′ R- 5'-CTCCTCCACACACAGAAGCA-3′	(Zieker *et al.*, 2005)
BCL11B	F: 5'-CTCTCACCCACGAAAGGCAT-3' R: 5'-GCACGCAGAGGTGAAGTGAT-3'	(Pinho *et al.*, 2012)
E4BP4	F-5'-CCAAGGGCCCCATCCATTC-3′ R-5'-GATGCCAGTGCTCCGATTTG-3'	(Vacca *et al.*, 2011)
EOMES	F: 5'-ACTGGTTCCCACTGGATGAG-3' R: 5'-CCACGCCATCCTCTGTAACT-3'	(Hertoghs *et al.*, 2010)
GATA-3	F: 5'-AGCACAGAAGGCAGGGAGTGT-3' R: 5'-TTCGCTTGGGCTTAATGAGGGGC-3'	(Pinho *et al.*, 2012)
Granzyme B	F: 5'-TGGGGGACCCAGAGATTAAAA-3' R: 5'-TTTCGTCCATAGGAGACAATGC-3'	(Morissette *et al.*, 2007)
HELIOS	F: 5'-ACACCTCAGGACCCATTCTG-3′ R: 5'-TCCATGCTGACATTCTGGAG-3′	(Cai *et al.*, 2009)
ID2	F-5'-CGGATATCAGCATCCTGTCC-3' R-5'-TCATGAACACCGCTTATTCAG-3'	(Cupedo *et al.*, 2009)
IFN-γ	F-5'-CCAGGACCCATATGTAAAAG-3' R-5'-TGGCTCTGCATTATTTTTC-3'	(Gober *et al.*, 2008)
IRF-2	F: 5'-CCTATGCAGAAAGCGAAACGACTGA-3′ R: 5'-TCGAGTCCCCATGTTGCTGAGGT-3′	(Pinho *et al.*, 2012)
KIR2DL1 (CD158a)	F-5'-GCAGCACCATGTCGCTCT -3′ R-5'-GTCACTGGGAGCTGACAC-3'	(Cooley *et al.*, 2007)
KIR2DL2/DL3 (CD158b)	F-5'-GGAGGGGGAGGCCCATGAAT-3′ R-5'-GTCGGGGGTTACCGGTTTTA-3'	(Cooley *et al.*, 2007)
NKG2A	F-5'-ACTGAACAGGAAATAACCTAT-3′ R-5'-ATGAGCTTCTCTGGAGCTGATC-3'	.(Arlettaz *et al.*, 2004)
NKG2C	F-5'-CTCATGGATTGGTGTGTTTCGT-3′ R-5'-CACTGTAAACGCAAATGCTTTACTTC-3'	Web resource: http://biowww.net/gene/gene-KLRC2.html
Perforin	F: 5'-CGCCTACCTCAGGCTTATCTC-3' R: 5'-CCTCGACAGTCAGGCAGTC-3	(Morissette *et al.*, 2007)
PU.1	F: 5'-TGTTACAGGCGTGCAAAATGGAAGG-3' R: 5'-CTCGTGCGTTTGGCGTTGGTATAGA-3'	(Bonadies *et al.*, 2010)
RORC	F-5'-AGTCGGAAGGCAAGATCAGA-3′ R-5'-CAAGAGAGGTTCTGGGCAAG-3'	(Ortega *et al.*, 2009)
T-BET	F: 5'-GGATGCGCCAGGAAGTTTCA-3' R: 5'-CTCTGGCTCTCCGTCGTTCA-3'	(Pinho *et al.*, 2012)
TOX	F: 5'-TATGTGCCAGCCAGCCAGTCCTA-3' R: 5'-TGGTCTGGGAGGGAAGGAGGAGTAA-3'	(Pinho *et al.*, 2012)

All primers were tested by simulated PCR using Amplify 3x program (Bill Engels, University of Wisconsin 2005, http://engels.genetics.wisc.edu/amplify/).

2.3 Methods

2.3.1 Overview

This project combines different techniques in order to reach the proposed aims. A summary of the general methodology is described in figure 2.1.

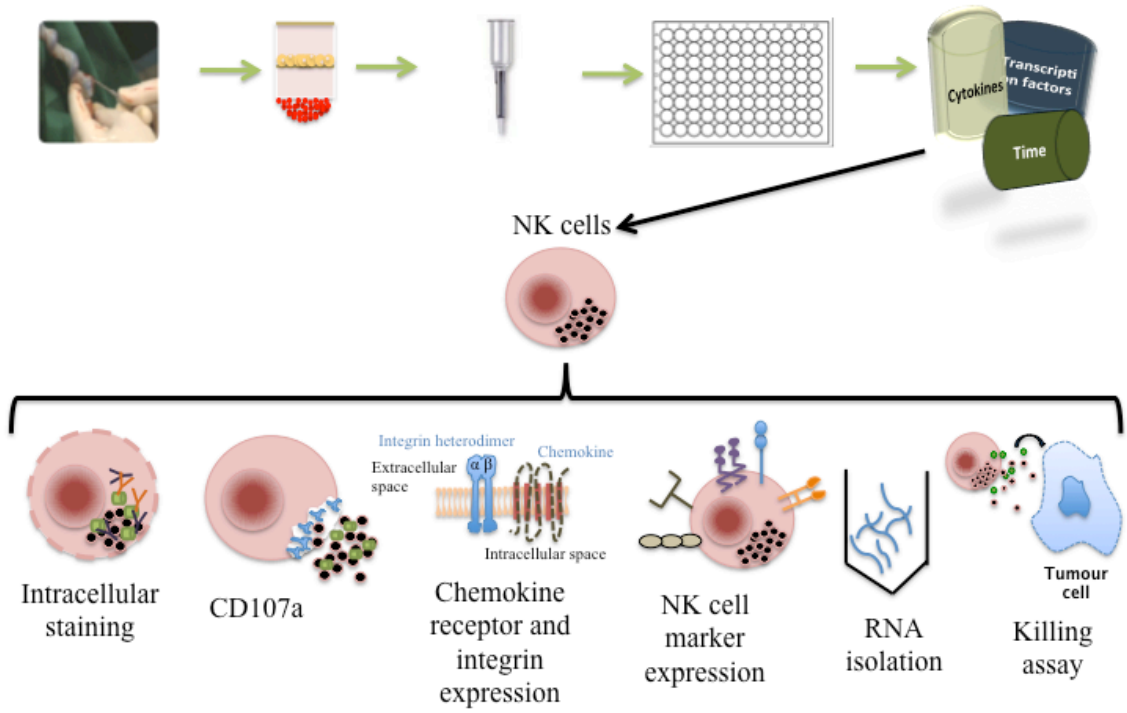


Figure 2.1. General methodology. Fresh CB was obtained and following ficoll separation, $CD34^+$ stem cells were isolated from CB mononuclear cells (CBMCs) and used either immediately or frozen for further use. Mobilised peripheral blood stem cells were kindly provided in frozen cryovials by Dr Kwee Wong. Cells were plated in 96 flat bottom well plates over irradiated EL08.1D2 cells and cultured with a mixture of cytokines for 4-5 weeks. Throughout the culture, generated NK cells underwent a series of analysis, including intracellular staining (perforin, granzyme B, and IFN-γ), degranulation assay by analysing CD107a expression, expression of chemokine receptors and integrins by flow cytometry, ^{51}Cr killing assay and finally weekly RNA isolation for molecular work.

2.3.2 Cell lines

All cell lines except the adherent feeder layer were grown in suspension at 37°C with 5% CO_2 using 25, 75 or 125 cm^2 flasks. The media for all suspension cell lines consisted of RPMI supplemented with heat inactivated FBS and 1% Penicillin/Streptomycin. The cell concentration was calculated using trypan blue to count only viable cells. Cells underwent a change of media every two to three days and were maintained at a concentration of between 0.3-1 x 10^6 cells/ml.

The embryonic liver cell line EL08.1D2 was cultured on gelatinised plates at 32°C with 5% CO_2. Bovine gelatine 0.1% was used to coat the flasks for 15-30 min at 37°C and then washed with 1X PBS. The cells were plated in the appropriate volume (12 ml for

75 cm^2, 24 ml for 125 cm^2 and 30 ml for 175 cm^2) of basic medium: 40.5% α–minimum essential medium (α-MEM), 50% myelocult, 7.5% FBS, with β-mercaptoethanol (50 μM), glutamax (2 mM), penicillin (100 U/ml) / streptomycin (100 U/ml), and hydrocortisone (10^{-6} M) containing 20% of conditioning media (0.2 nm filtered supernatant from previous cultures). Cells were seeded at 4000 cells/cm^2 and cultured until 95% confluence was reached. To detach and count the cells, the cells were trypsinised by washing the flask with 1X PBS and adding enough 0.25% Trypsin with EDTA to cover the surface of the flask (2, 5, and 7 ml for 75 cm^2, 125 cm^2 and 175 cm^2 respectively). After 4 min of incubation at 37°C and visual observation under the microscope of the detached cells, warm RPMI media with 10% FBS (basic media) was added to stop the trypsin's action. Cells were collected and washed once with RPMI and centrifuged at 1200 rpm for 5 min. Prior to co-culture with progenitor cells, EL08.1D2 cells were irradiated for 7 min (3000 rads), plated in 96 well gelatin coated plates and left overnight to allow cell adhesion.

2.3.3 Cell separation

2.3.3.1 Mononuclear cell separation

To obtain CBMCs, heparinised CB was mixed with an equal volume of transport media (table 2.5) and incubated at room temperature. After 45-60 min, CBMCs were isolated by density centrifugation through Ficoll—Paque PLUS (2000 rpm for 30 min without brake). The mononuclear cell layer was removed using a Pasteur pipette and washed twice with RPMI 1640. When needed, red cell lysis was performed with 1 ml of BD pharma lyse buffer for 4 min and an additional washing step was performed (10 min at 1600 rpm at room temperature). Cells were resuspended at a concentration of 2 x 10^6 cells/ml in basic media and counted using Turk's (genetian violet and acetic acid 6%) staining reagent.

When healthy control subjects were needed, isolated PBMCs were used. PB was mixed with an equal volume of RPMI medium and incubated at room temperature. Using 50 ml falcon tubes, the diluted blood was added to Lympholyte in a 2:3 ratio (3 ml of Lympholite and 6 ml of diluted blood). The sample was centrifuged at 2000 rpm for 30 min at room temperature without brake. Next, PBMCs were isolated using the same procedure as CBMCs including washing, red cell lysis and cell counting.

2.3.4 Freezing and thawing of cells

Freezing cells

Cells were resuspended on ice using a freezing solution (table 2.5). Cell lines were cryopreserved in liquid nitrogen in vials containing 5 x 10^6 cells in 1.8 ml final volume

(2.77 x 10^6 cells/ml). Isolated stem cells were frozen in the same solution at a concentration of 1 x 10^6 cells/ml).

Thawing cells

Cryovials were thawed in a 37°C water bath incubator. The freezing medium containing the cells was taken out using a Pasteur pipette and added to a 15 ml falcon tube containing 10 ml of warm basic media. Cells were centrifuged for 5 min at 1200 rpm and later resuspended in 10 ml of basic media. For isolated $CD34^+$ or $CD133^+$ stem cells a thawing buffer was used. Criovials were thawed in the 37°C water bath incubator and the mixture containing cells in freezing medium was added to 1 ml of thawing buffer. Next, 10 ml of basic media was added, cells were then centrifuged for 5 min at 1200 rpm and then resuspended in 10 ml of basic media for enumeration.

2.3.5 Cell isolation

2.3.5.1 Natural Killer cell isolation

NK cells were isolated using a negative selection kit (table 2.3). Cell labelling was done following the manufacturer's instructions except for the final step in which the cells were resuspended in 5 ml instead of 500 µl of MACS buffer. Magnetic separation was carried out using LS columns (table 2.6). Cells were placed onto the magnetic columns and two washes of 7 ml of MACS buffer were performed. Unlabelled cells were collected in 50 ml falcon tubes, centrifuged at 1200 rpm for 10 min and counted using trypan blue.

The mean of the purity (percentage) and standard deviation of the NK cell isolations was 85.2 ± 6.2% for PB and 89.4 ± 6.2% for CB (figure 2.2).

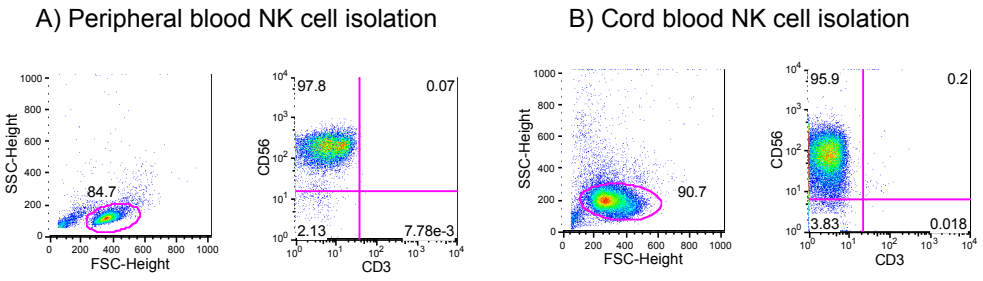


Figure 2.2. NK cell isolations. NK cells were isolated from PBMCs or CBMCs by negative selection. A representative plot of isolated NK cells from PBMCs (A) and CBMCs (B) is shown; to determine purity, lymphocytes were gated according to forward versus side scatter (left panel) and from that gate the expression of CD56 versus CD3 was determined (right panel). NK cells are characterised by the expression $CD56^+$ and absence of $CD3^-$.

2.3.5.2 Stem cell isolation:

Frozen CD34$^+$ PBSC samples were kindly provided by Kwee Yong (UCL, Cancer Institute). Although fresh PBSC are regularly used for NK cell generation *in vitro*, only cryopreserved samples were available for this study. To isolate CB CD34$^+$ cells, the CD34 MicroBead kit was used (table 2.3). The first isolations using the protocol provided by the manufacturer yielded very low purity (around 60%), therefore the protocol was modified according to a published protocol involving two labelling steps to improve purity (Jaatinen and Laine, 2007). All solutions were cold to avoid capping of antibodies. After CBMCs collection, 300 μL of labelling buffer (Table 2.5) with 20% AB serum was added per 10^8 cells (figure 2.3). The remaining labelling and magnetic separation procedure was performed according to the protocol. Isolated cells were collected, resuspended in basic media and counted with trypan blue.

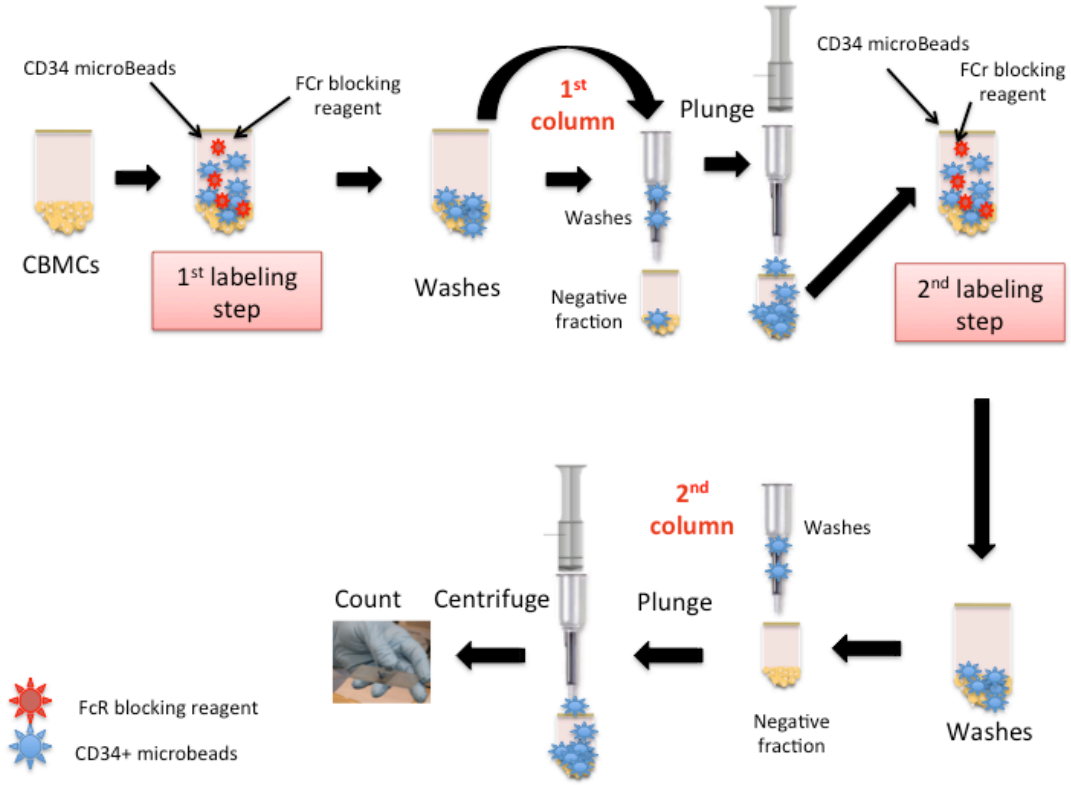


Figure 2.3. CD34 isolation. CBMCs were incubated with CD34$^+$ MicroBeads plus FcR blocking reagent. After washing, cells were placed on a magnetic LS column and washed four times. Using the plunger provided by the manufacturer, cells were collected and a second labelling with MicroBeads and blocking reagent was performed. Cells were applied to a second column (MS) and after washes; cells were collected, resuspended and counted for further use.

The purity of the isolations was analysed following the ISHAGE (International Society of Hematotherapy and Graft Engineering) guidelines (Sutherland *et al.*, 1996). Figure 2.4 shows a representative CD34 isolation.

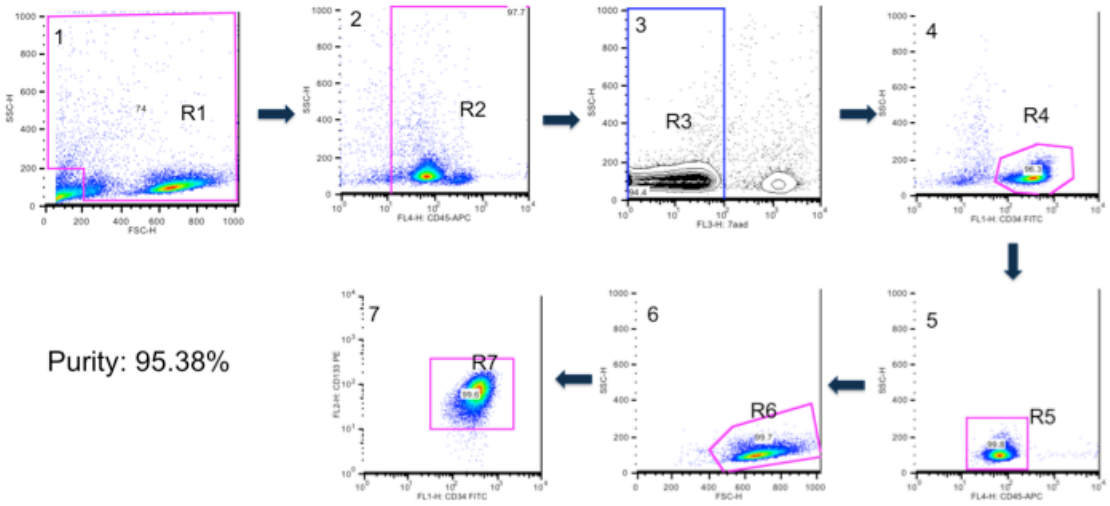


Figure 2.4. Stem cell isolation from umbilical cord blood. 1) Forward versus side scatter analysis of CD34 isolated cells. 2) Sample stained with anti-CD45 APC gated in R1, stem cells are characterised by being $CD45^{low}$. 3) 7AAD staining of R2 gate, gate R3 represents live $CD45^{+}$ cells. 4) $CD34^{+}$ versus side-scatter analysis of $CD45^{+}7AAD^{-}$ cells. 5) Region 5 represents $CD34^{+}CD133^{+}7ADD^{-}$ cells. 6) Gate R6 represents the lymphoblast gate from R5. 7) $CD133^{+}$ versus $CD34^{+}$ analysis showing live lymphoblastic double positive cells. The purity was calculated from the total events of double positive $CD133^{+}$ and $CD34^{+}$ divided by the total events of live cells: (R7/R3)*100.

The purity of the performed isolations was analysed following the ISHAGE guidelines. The mean of the purity (percentage) and standard deviation of the $CD34^{+}$ isolations was 93.2 ± 6%.

2.3.6 Culture conditions

2.3.6.1 Culture conditions for Natural Killer cell differentiation in vitro

The Basal Culture Medium consisted of 2:1 (vol:vol) ratio of DMEM with 4.5g/l glucose, L-glutamine, and sodium pyruvate/Ham's F12 Medium. This medium mixture was then supplemented with: 50 µM β mercaptoethanol, 50 µM ethanolamine, 20 mg/l ascorbic acid, 50 µg/l sodium selenite, 1% penicillin + streptomycin and 20% heat-inactivated human AB serum. The media was kept at 4°C for up to one month.

Two different cytokine mixes were used for NK cell cultures. In both cases, a 3 week period was maintained under the same condition (termed all cytokines): 10 ng/ml IL-15, 5 ng/ml IL-3 (only for the first week), 20 ng/ml IL-7, 20 ng/ml c-kit ligand (SCF) and 10 ng/ml Flt3 ligand. At week 3, all cytokines condition remains exactly the same for the rest of the culture time. Modified protocol: removal of all the factors and cytokines except for IL-15, a new concentration was assigned, 50 ng/ml (termed only IL-15). Cultures underwent weekly semi-depletion of fresh medium supplemented with the corresponding cytokines.

2.3.6.2 CD33 blocking

In order to block CD33 on NK cells the anti-Human CD33 from eBiosciences (clone WM-53) antibody was used. NK cells were pre-incubated for 30 min with anti-CD33 at a concentration of 5 µg/ml (Hernandez-Caselles *et al.*, 2006) for CD107a, intracellular staining for IFN-γ and ^{51}Cr killing assays. Cells were not washed and the antibody concentration was maintained during the assay.

2.3.6.3 IL-12 incubation

IL-12 was used to augment NK cell cytotoxicity and promote maturation. A concentration of 20 ng/ml for a period of 4, 24 and 40 h was used. Cells were plated in 96 well plates at a final volume of 200 µl and incubated for 4, 24 and 40 h. The cells were then collected into a 50 ml falcon tube and washed with RPMI media before being used in assays.

2.3.7 Characterisation by flow cytometry

2.3.7.1 Cell surface staining

Characterisation of NK cells was performed by flow cytometry. Prior to staining, Fc receptors were blocked with blocking buffer (Table 2.5) for 15 min at room temperature to inhibit the non-specific binding of the Fc portion of the fluorochrome-conjugated mAbs. After blocking, cells were incubated in the dark at 4°C for another 10 min (except for CXCR4 and CXCR7 for which 45 min incubation time was needed), washed and resuspended in FACS buffer. A FACS calibur (Becton & Dickson Oxford UK) was used to acquire data and FlowJo (Tree Star Inc., OR, USA) software for analysis.

2.3.7.2 Functional assays

2.3.7.2.1 Intracellular assay:

2.3.7.2.1.1 IFN-γ

A total of 2 x 10^5 NK cells were transferred to 96 U shaped well plates and centrifuged for 3 min at 1800 rpm. Cells were incubated without stimulus or with cell lines (K562 or Raji ratio 1:1) or PMA 100 ng/ml and 1 µg/ml Ion as positive control for 1 h at 37°C, 5% CO_2, 96% humidity in the incubator. After 1 h incubation, Golgi Stop (4 µl for every 6 x 10^6 cells) was added to each well according to the manufacturer's instructions and incubated for a further four hours. Cells were then blocked with mouse serum before antibodies were added (anti-CD56, -CD16 and -CD3) in 50 µl of labelling buffer and incubated for 10 min at 4°C in the dark. Next, cells were resuspended in 100 µl of

permabilisation/fix buffer (BD cytofix/cytoperm plus, UK) and incubated for 5 min at room temperature. Following permeabilisation, cells were blocked, washed and resuspended in 50 µl of anti–IFN-γ antibody or the appropriate isotype control made in Perm/Wash buffer and incubated for 1 h at room temperature in the dark. Cells were washed twice with and resuspended in Perm/Wash buffer before immediate analysis by flow cytometry.

2.3.7.2.1.2 Perforin and Granzyme B

A total of 2 x 10^5 NK cells were transferred to 96 U shaped well plates and were centrifuged 3 min at 1800 rpm. Cells were blocked with mouse serum before antibodies were added (anti-CD56, -CD16 and -CD3) in 50 µl of labelling buffer and incubated for 10 min at 4°C in the dark. Next, cells were resuspended in 100 µl of permabilisation/fix buffer (BD cytofix/cytoperm plus, UK) and incubated for 5 min at room temperature. Following permeabilisation, cells were blocked, washed and resuspended in 50 µl of antibody anti–granzyme B, anti-perforin or the appropriate isotype control made in Perm/Wash buffer and incubated for 1 h at room temperature in the dark. Cells were washed twice with and resuspended in Perm/Wash buffer and immediately analysed by flow cytometry.

2.3.7.2.2 Degranulation assay CD107a

A total of 2 x 10^5 NK cells resuspended in 200 µl of complete medium were added to 96 U shaped well plates. Cells were incubated for 2 h at 37°C, 5% CO_2, 96% humidity in the incubator in the presence of only media, K562 or Raji (1:1 ratio), or PMA 100 ng/ml and 1 µg/ml Ionomycin as a positive control. Cells were then blocked for 15 min at room temperature with blocking buffer, centrifuged and stained for CD56, CD3 and CD16 in staining buffer for 10 min at 4°C. The cells were then washed and stained with anti-CD107a or the appropriate isotype control for 45 min at 4°C. Finally, the cells were washed, resuspended in 1X PBS containing 2% FBS and 2 mM EDTA before flow cytometry analysis.

2.3.7.2.3 ^{51}Cr release assay

Prior to the assay, the cell line K562 was sub-cultured and maintained at log phase (0.5-1 x 10^6 cells/ml). K562 cells were washed with 1X PBS and then labelled for 45 min with 100 µCi / 1 x 10^6 at 37°C, 5% CO_2, 96% humidity in the incubator. Subsequently, cells were washed twice with 1X PBS. Effector cells were plated in U-bottom plates at effector-to-target ratios 1:1, 5:1 and 10:1 in triplicate in RPMI media

supplemented with basic media. Maximum Chromium release was obtained by incubating target cells with 1% Triton X in 1X PBS, while the minimum release was obtained by incubation with basic media. After 4 h co-culture at 37°C, cells were centrifuged at 1400 rpm for 4 min and 30 µl of supernatant was collected over 96 well plates and left overnight to dry. Once dried, 30 µl of scintillation solution was added in each well and the 96 well plate was read with the 1450 Micro beta counter. The percentage of specific lysis was calculated as (experimental release – spontaneous release)/(maximum release – spontaneous release) x 100.

2.3.7.2.4 ADCC

Prior to the assay, the murine cell line P815 was sub-cultured and left at log phase (0.5-1 x 10^6 cells/ml). P815 cells were washed with 1X PBS and then labelled for 45 min with 100 µCi / 1 X 10^6 at 37°C. The cells were then washed twice with 1X PBS. P815 target cells were incubated for 30 min at 37°C with anti-CD16 (1 µg/ml) or the respective isotype at the same concentration. Finally, cells were washed twice with 1X PBS and the ^{51}Cr release assay was performed as previously described.

2.3.8 ELISA

2.3.8.1 IFN-γ

To determine the amount of IFN-γ in the samples, the Human Interferon–gamma Ready-set-go!® sandwich ELISA kit from eBioscience was used. Supernatants of 0.2 x 10^6 NK cells stimulated (PMA, K562 or Raji cell lines ratio 1:1) and non-stimulated cells incubated at 37°C, 5% CO_2, for 2 h were used. IFN-γ was detected following the manufacturer's instructions using the KC junior software (Bio-Tek, Vermont, USA).

2.3.8.2 TNF- α

To determine the concentration of TNF-α in cell supernatants, the Human TNF alpha ELISA Ready-SET-Go!® kit from eBioscience was used. Supernatants of 0.2 x 10^6 NK cells stimulated (PMA, K562 or Raji cell lines ratio 1:1) and non-stimulated cells incubated at 37°C, 5% CO_2, for 2 h were used. TNF-α was detected following the manufacturer's instructions using the KC junior software (Bio-Tek, Vermont, USA).

2.3.9 Molecular characterisation of cord blood Natural Killer cells, peripheral blood Natural Killer cells and Natural Killer cells generated *in vitro*

2.3.9.1 RNA

RNA extraction was performed using the RNAeasy Minikit according to the manufacturer's instructions except for the final step in which RNA was dissolved from the column with 30 µl (two spins of 15 µl each, to increase yield) of RNAse free water and immediately placed onto ice. The RNA concentration was measured using the Nano Drop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., BE, USA) and then stored at -80°C.

2.3.9.2 Reverse transcription

Extracted RNA was used as a template to create complimentary DNA (cDNA). 200 ng of RNA were added to a PCR tube (Thermo Scientific, USA) together with distilled H_20 (d.H_20), 2 µl of random primers and 1 µl of dNTPs. The mixture was incubated in the Eppendorf Mastercycler thermocycler (Eppendorf USA via Fisher Loughborough UK) for 5 min at 65°C and then 5 min on ice. Next, 4 µl of Buffer 5X were added, 2 µl of DTT (0.1M), and 1 µl of RNase inhibitor. This mix was incubated for 10 min at 25°C and then 2 min at 42°C. 1 µl of Superscript III was added to the tube and an additional incubation of 50 min at 42°C was performed. Lastly, a 15 min at 70°C step was made. The cDNA stock was diluted by the addition of 40 µl of d.H_20 and used immediately or stored at -20°C.

2.3.9.3 Real time PCR

All PCR preparation work was performed on ice. The primer concentrations used are shown in table 2.10. The final volume in all reactions was 25 µl, containing 10 µl of PCR precision 2X precision Mix, the appropriate amount of forward and reverse primers according to the concentration used, 5 µl of cDNA and finally the d.H_20 was adjusted according to the primer concentration.

Table 2.10. Real time PCR primer concentrations

Primer	HSC and CB NK cells (nM)	PB NK cells (nM)
2B4 (CD244)	900	900
BCL11B	300	300
E4BP4	300	300
EOMES	200	300
GATA-3	300	300
Granzyme B	300	300
HELIOS	600	600
ID2	300	300
IFN-γ	300	300
IRF-2	300	300
KIR2DL1 (CD158a)	600	600
KIR2DL2/DL3 (CD158b)	600	600
NKG2A	900	600
NKG2C	900	600
Perforin	900	900
PU.1	300	300
RORC	900	900
T-BET	300	300
TOX	300	300

All reactions (except E4BP4) were performed using the following programme, 2 min 50 °C, 10 min 95 °C, and 50 cycles of 15 sec 95 °C and 1 min 60 °C. For E4BP4 we used: 2 min 50 °C, 10 min 95 °C, and 40 cycles of 15 sec 95 °C and 30 sec 60 °C.

2.3.10 Statistics

Statistical comparisons were performed with GraphPad Prism software (GraphPad Software) using the nonparametric Mann–Whitney test or paired t-test. Results are presented as means ± standard deviation (SD), $p<0.05$ were considered statistically significant. For comparisons between fresh and frozen CBSC cultures, a blue asterisk (*) is shown if there was any statistical significance, whereas a black asterisk is shown between the comparisons of frozen CBSC and PBSC (*). In some instances where only “fresh CBSC” and “all cytokines” legends appear, fresh CBSC is the same as “only IL-15”. * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

Chapter 3: Production of Natural Killer cells *in vitro* from haematopoietic stem cells

3.1 Introduction

Different sources of HSC have been used to generate NK cells *in vitro* including BM precursors (Miller *et al.*, 1994; Mrozek *et al.*, 1996), hESC (Woll *et al.*, 2005; Woll *et al.*, 2009), PBSC (Yoon *et al.*, 2010; Zamai *et al.*, 2012) and CBSC (Spanholtz *et al.*, 2010; Spanholtz *et al.*, 2011a; Dezell *et al.*, 2012; Lehmann *et al.*, 2012). Each of these sources has their own advantages and disadvantages.

BM has been a preferred source of HSC to study NK cell development but not particularly to produce NK cells for immunotherapy. Numerous studies used BM to generate NK cells *in vitro* in order to identify the precursors that give rise to NK cells in humans (Miller *et al.*, 1994; Haddad *et al.*, 2004; Grzywacz *et al.*, 2011; Vacca *et al.*, 2011) and the cytokines and microenvironment requirements for these precursors to develop into NK cells (Mrozek *et al.*, 1996; Kusadasi *et al.*, 2001; Sivori *et al.*, 2003). To our knowledge, there are currently no clinical protocols using BM precursors in order to generate NK cells for immunotherapy. The two major drawbacks to develop NK cells from BM precursors are the nature of its invasive collection delivering low HSC per sample (Korbling and Anderlini, 2001) and stringent HLA-matching requirements.

The use of hESC for NK cell generation purposes has been tested with promising results (Woll *et al.*, 2005; Woll *et al.*, 2009). However, the nature of these cells is what has stopped further developments in this area as destroying 5-7 days old embryos for immunotherapeutic purposes would be unethical. Nevertheless, hESC cell lines have been developed and are currently tested for NK cell production *in vitro* but additional characterisation is needed and, notably, such an approach will have to overcome safety requirements in order to be translated to the clinic.

As mentioned above, BM and hESC are great sources of HSC but carry ethical predicaments or involve invasive collection procedures. The introduction of G-CSF into the clinics has allowed the collection of HSC in a safe and less invasive procedure. A few studies report using PBSC as a HSC source to produce NK cells *in vitro* (Giuliani

et al., 2008; Zamai *et al.*, 2012). In addition, CB is another promising HSC source providing convenient collection procedures and off-the-shelf availability. The number of units available worldwide (http://www.bmdw.org/) (Mayani, 2011) along with the less stringent HLA-matching makes CBSC an optimal source for NK cell generation *in vitro*. However, one study in particular reported unfavourable outcomes regarding the use of CBSC for this approach (Woll *et al.*, 2009). The study performed by Woll *et al*. showed that hESC generated NK cells with a mature phenotype and a potent cytolytic activity as compared to a mix of immature/mature cells with less anti-tumour activity derived from CB (Woll *et al.*, 2009). The need for defining whether CBSC or PBSC are better HSC sources for this approach is critical.

The use of cryopreserved cells will enable the off-the-shelf availability of the cell products in clinical settings. Different studies showed that expansion of frozen HSC is often poor (Boissel *et al.*, 2008a) with a decreased stem cell count and viability (Beshlawy *et al.*, 2009), while others report that frozen CBSC could be used to generate NK cells (Spanholtz *et al.*, 2010) because of their high proliferative and clonogenic capacity, *ex vivo* expansion (Lu *et al.*, 1996; Moezzi *et al.*, 2005), and engraftment potential in immunocompromised mice (Broxmeyer *et al.*, 2003). There have been several studies using fresh or frozen CBSC with the direct or indirect aim of generating NK cells *in vitro* (Grzywacz *et al.*, 2006; Spanholtz *et al.*, 2010; Spanholtz *et al.*, 2011a; Dezell *et al.*, 2012; Pinho *et al.*, 2012); however a comprehensive study of the impact of using both sources of HSC for generating NK cells for immunotherapy has not yet been performed.

A variety of protocols for NK cell generation have been published (reviewed by Luevano *et al*.(Luevano *et al.*, 2012b)). We chose to use the protocol developed by Grzywacs *et al*. (Grzywacz *et al.*, 2006) for this study in which a combination of SCF, IL-7, IL-15, FLT3 ligand and the feeder layer EL08.1D2 was used. Because previous reports have shown that IL-15 plays a key role for NK cell development and that the remaining factors (SCF, IL-7 and FLT3 ligand) will only increase NK cell precursor frequencies (Cavazzana-Calvo *et al.*, 1996; Mrozek *et al.*, 1996), we decided to test the withdrawal of all factors except IL-15 at week 3 and increased IL-15 concentration (from 20 ng/mL to 50 ng/mL) as this modification decreased the amount of cytokines used and therefore reduced costs.

In this work a comparison between CBSC and PBSC as HSC sources for NK cell generation *in vitro* because of their advantages over hESC and BMSC was performed. As the use of fresh samples is commonly used in protocols generating NK cells, the

first aim was to analyse the impact of using a published protocol (referred to as all cytokines) (Grzywacz *et al.*, 2006) and a modified protocol (only IL-15 for the last two weeks, referred to as only IL-15) using fresh CBSC. Next, the use of fresh versus frozen CBSC and CBSC versus PBSC as HSC sources for NK cell generation *in vitro* was investigated. This chapter describes a detailed study of NK cell generation including NK cell fold expansion, NK cell number retrieved, expression of lymphoid and myeloid lineage markers and finally a comprehensive characterisation of NK cell development including a molecular profile of transcription factors.

3.2 Results

3.2.1 Frozen cord blood stem cells cultures generate higher Natural Killer cell numbers than fresh cord blood stem cell cultures and peripheral blood stem cell cultures

During this thesis, CB $CD34^+$ cells (CBSC) were isolated using MACS as described in section 2.3.5.2, mobilised PB $CD34^+$ cells (PBSC) were kindly provided by Dr Kwee Yong. CBSC (fresh or frozen) and PBSC (frozen) were cultured for up to 5 weeks and fold expansion, NK cell number, viability and NK cell yield were assessed on a weekly basis. We investigated the proliferation of the seeded cells by enumeration of the total cell number per well. A higher fold expansion in frozen CBSC cultures at day 21 and 28 ($p<0.05$) compared to fresh CBSC and PBSC cultures was observed, nonetheless, at day 35 only a difference in fold expansion between fresh and frozen CBSC cultures was found (189.7 ± 88.5 versus 520.1 ± 134.3, respectively, $p<0.05$, figure 3.1A). When using fresh CBSC, all cytokines or only IL-15, no differences were observed in fold expansion or NK cell numbers (figure 3.1B and 3.2B respectively). In addition, higher NK cell numbers in frozen CBSC cultures than fresh CBSC and PBSC cultures were found (day 28 and 35, $p<0.05$) (figure 3.2A).

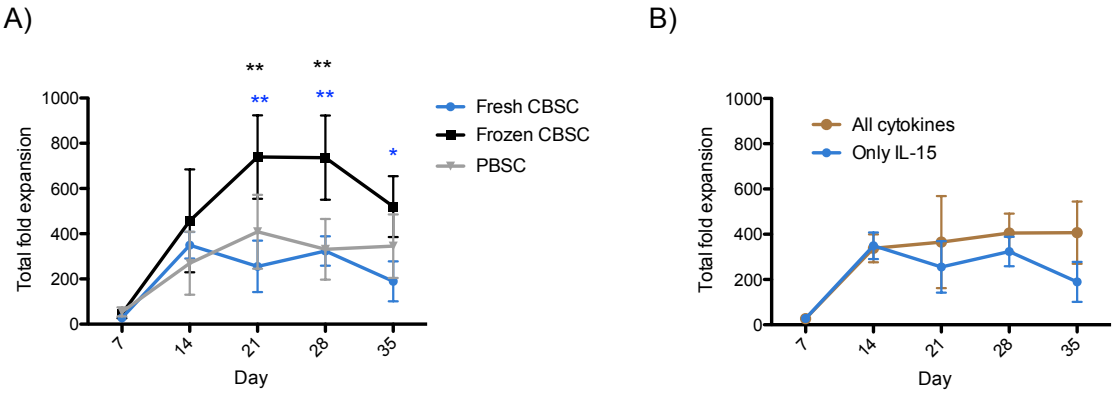


Figure 3.1. NK cell production from HSC cultures *in vitro*. A) Median values ± SD of the total fold expansion of fresh CBSC (n=5), frozen CBSC (n=9) and PBSC (n=6) cultures at different time points. B) Total fold expansion of CBSC cultures using all cytokines (n=3) or only IL-15 (n=3). Total amount of cells were counted and fold expansion was calculated by division of this number by the initial number of HSC seeded (total number cells/500 initial cells)* 100. Mann-Whitney test was performed, *** p<0.05, ** p<0.01, ***p<0.001**.

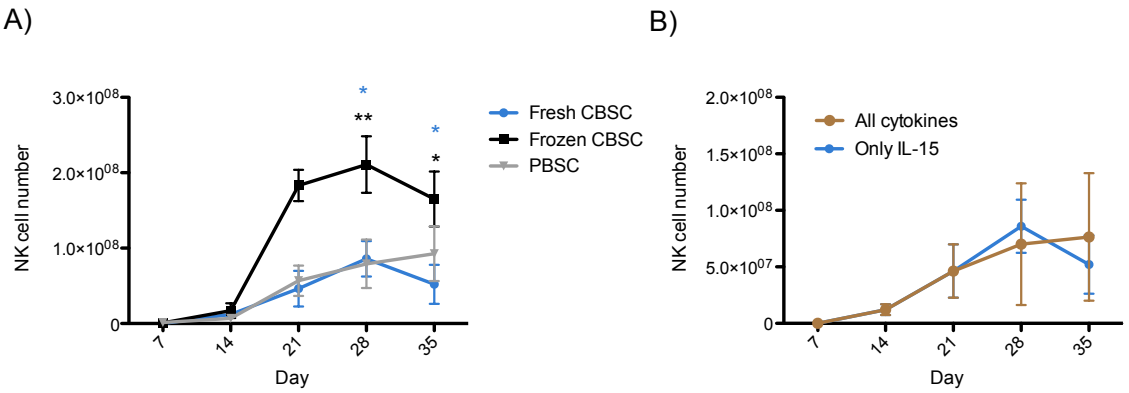


Figure 3.2. Total NK cell number from HSC cultures. A) $CD3^{-}CD56^{+}$ cells from fresh CBSC (n=3), frozen CBSC (n=9), PBSC (n=6) cultures and B) from CBSC cultures using all cytokines (n=3) or only IL-15 (n=3). Cell numbers were calculated as follow: (initial amount of stem cells seeded [0.288 x 10^6] x fold expansion [for each time point] x NK cell percentage)/100. Mann-Whitney test was performed, * p<0.05, ** p<0.001, ***p<0.001.

In order to monitor viability, staining for 7AAD was performed on a weekly basis. All the cultures displayed good viability with no significant differences between HSC cultures (figure 3.3).

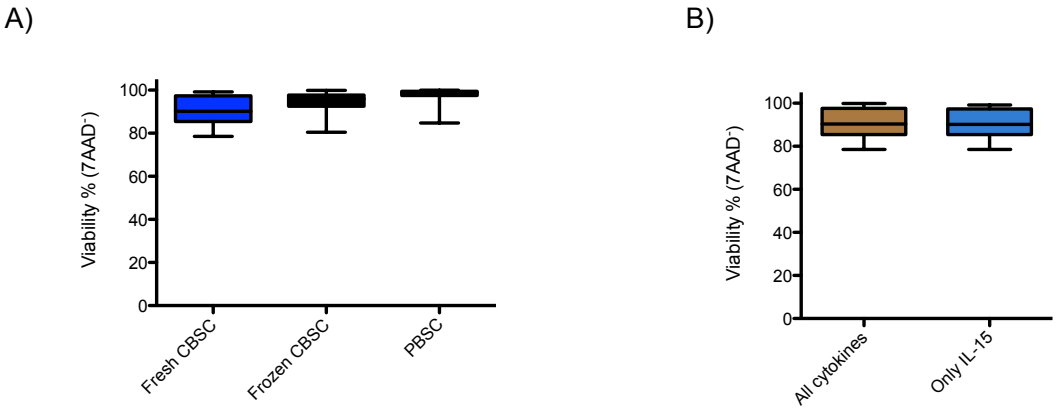


Figure 3.3. Cell viability in HSC cultures. A) Percentage of $7AAD^{-}$ cells from the lymphocyte gate of fresh CBSC (n=3), frozen HSC (n=9), PBSC (n=6) cultures and B) CBSC cultures using all cytokines (n=3) or only IL-15 (n=3) for all time points.

NK cells are characterised by the expression of CD56 cell surface marker and the absence of CD3; we analysed the expression of these markers on a weekly basis to assess NK cell yield in each culture. Figure 3.4A depicts NK cell production from fresh and frozen CBSC and PBSC cultures throughout the culture. Figure 3.4B shows NK cell production from fresh CBSC cultures using all cytokines or only IL-15 throughout the culture. A rapid increase in NK cell percentage from day 14 to day 21 was observed; by day 35 the majority of the cells in culture were NK cells. Additionally, the cultures displayed different NK cell subsets as analysed by flow cytometry, especially

in NK cells derived from fresh CBSC cultures (CBSC-NK cells, figure 3.4). Fresh CBSC-NK cells exhibited two distinct populations according to CD56 expression, similar to $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets. These two populations were also present in frozen CBSC cultures, but the majority of the cells appeared to be $CD56^{dim}$ NK cells. Interestingly, defined NK cell subsets in PBSC cultures were not observed. In addition, the mean fluorescent intensity (MFI) of CD56 on the generated NK cells was analysed. Although difference between cultures was not found, a trend for higher CD56 MFI in fresh CBSC cultures was observed (figure 3.5).

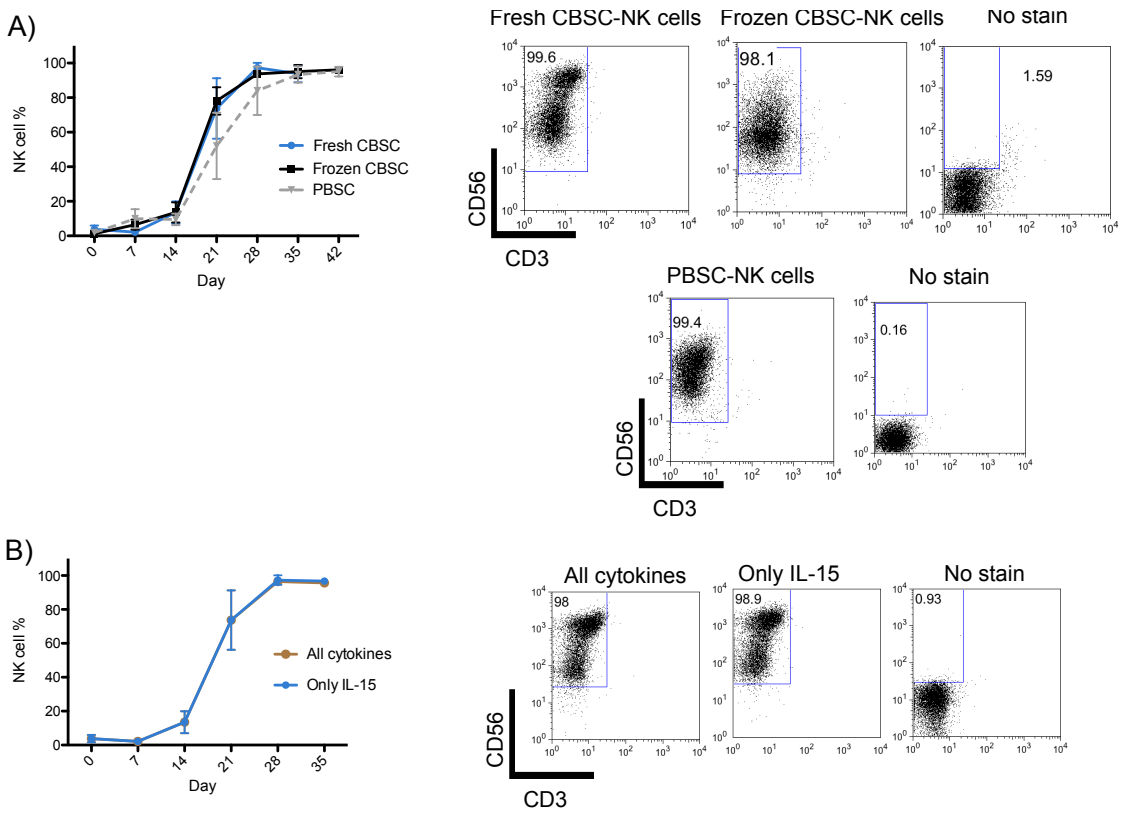


Figure 3.4. NK cell yield in HSC cultures. A) $CD3^{-}CD56^{+}$ NK cell percentages from the lymphocyte gate at different time points for fresh CBSC (n=3), frozen CBSC (n=9) and PBSC (n=6) cultures. The right panel shows a representative plot of CD56 *versus* CD3 expression for the aforementioned samples. B) Graph shows $CD3^{-}CD56^{+}$ cell percentages from the lymphocyte gate at different time points for CBSC cultures using all cytokines (n=3) or only IL-15 (n=3). The right panel shows a representative plot of CD56 *versus* CD3 expression for CBSC cultures using all cytokines (n=3) or only IL-15 (n=3).

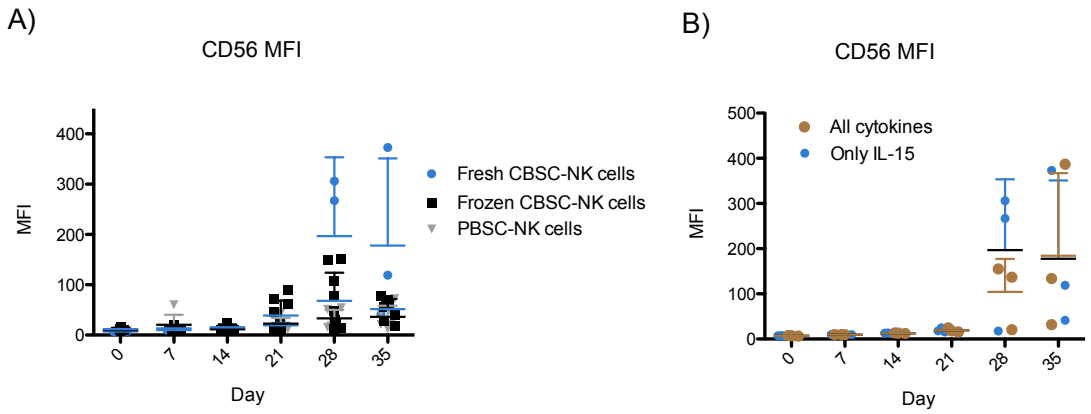


Figure 3.5. CD56 median fluorescent intensity. A) Median fluorescent intensity (MFI) of CD56 at different time points for fresh CBSC (n=3), frozen CBSC (n=9), PBSC (n=6) cultures and B) CBSC cultures using all cytokines (n=3) or only IL-15 (n=3).

CD56 acquisition on the generated NK cells was very similar between CBSC cultures and slower in PBSC cultures (figure 3. 6). At day 21 only 52.1 ± 19.2% of the cells were $CD56^{+}$ in PBSC cultures, compared to 78.2 ± 7.9% for frozen CBSC cultures and 73.7 ± 17.5% for fresh CBSC cultures. Nevertheless, CD56 expression was similar in all cultures by day 35 (figure 3.6). In summary, frozen CBSC showed a robust NK cell production *in vitro* exhibiting good viability and yielding higher NK cell numbers than other HSC sources.

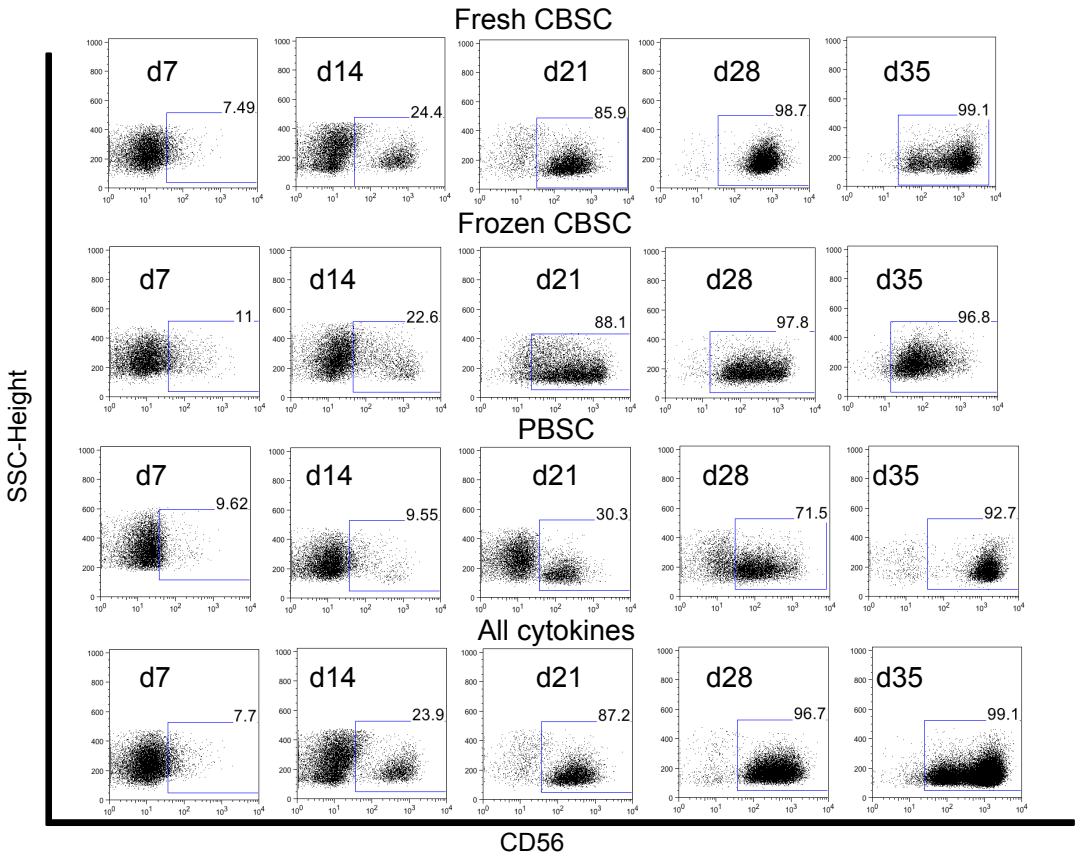


Figure 3.6. CD56 receptor acquisition in HSC cultures. A representative FACS plot of SSC-Height *versus* CD56 from the lymphocyte gate is shown from days 7-35 for fresh CBSC, frozen CBSC, PBSC and fresh CBSC cultures using all cytokines.

3.2.2 Absence of T, NKT and B cells in haematopoietic stem cell cultures

During the culture period, the presence of different cell lineages was analysed. The cytokine cocktail used in this study included IL-7, which is known to be important for T cell development (Puel *et al.*, 1998). Several conditions like GvHD or Epstein-Barr-virus-associated lymphoproliferative disease could be induced due to the presence of allogeneic T or B cells, respectively (Miller *et al.*, 2005). Therefore, having high purity during the culture is critical for adoptive immunotherapy. The presence of B cells (CD19) and T cells (CD3 and CD8) at different time points in the different HSC cultures was monitored using flow cytometry. Very low percentages of T cells ($CD3^+$, 0.9% ± 1.7), CTL ($CD8^+$, 5.7% ± 3.1) and B cells (0.7% ± 1) were found in each culture (figure 3.7).

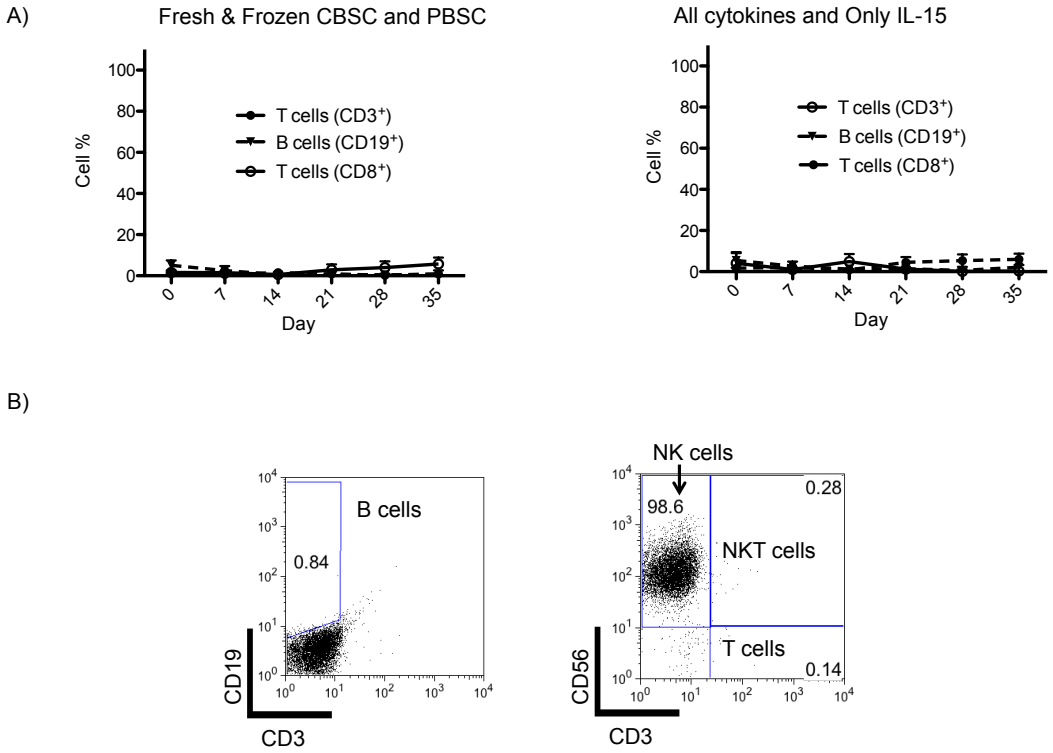


Figure 3.7. Different cell lineages in HSC cultures. Cells were stained with anti-CD3, -CD8 and -CD19. A) The graph depicts the mean and SD of $CD8^+$, $CD3^+$ and $CD19^+$ cells in all cultures from the lymphocyte gate: fresh CBSC (n=3), frozen CBSC (n=9), PBSC (n=6) cultures and CBSC cultures using all cytokines (n=3) or only IL-15 (n=3) at different time points. B) Flow cytometric analysis of a representative sample of frozen CBSC cultures at day 35. The left panel shows the FACS plots from the lymphocyte gate of CD3 *versus* CD19 and the right panel CD3 *versus* CD56.

In summary, the data indicates that during the culture of HSCs, the majority of cells develop into NK cells with very low percentages of T or B cells present throughout the culture.

3.2.3 Absence of monocytes in haematopoietic stem cell cultures

Some groups have reported the generation of monocytes ($CD14^+$) using *in vitro* culture systems to produce NK cells (Spanholtz *et al.*, 2010; Lehmann *et al.*, 2012). Therefore the screening in each culture for the presence of $CD14^+$ cells was performed. Interestingly, a transitory expression of CD14 on days 7 and 14 only in frozen cultures was observed (Figure 3.8, CBSC and PBSC). Sconocchia *et al*. have shown the existence of NK cells with monocyte-like properties (Sconocchia *et al.*, 2005a). These cells exist in PBMCs in very low frequency, around 1.2%, and can be generated *in vitro* from CBSC. This subset exhibits monocyte characteristics and is $CD56^{low}$, $CD33^+$, $HLA\text{-}DR^+$, $CD11b^{high}$ and $CD14^+$. Thus, the co-expression of CD14 and CD56 on the

generated NK cells was analysed. Figure 3.9 shows that the generated NK cells did not co-express CD56 and CD14 at day 35.

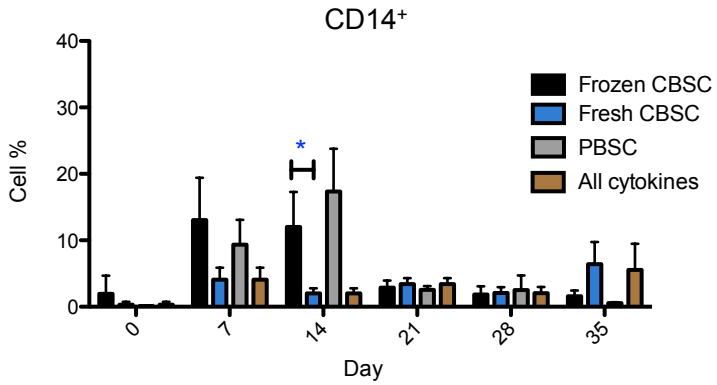


Figure 3.8. CD14 expression in HSC cultures. Graphs shows the mean cell percentage and SD values of $CD14^+$ cells from the lymphocyte gate for fresh CBSC (n=3), frozen CBSC (n=9), PBSC (n=6) cultures and CBSC cultures using all cytokines (n=3) at different time points. Mann-Whitney test was performed, * p<0.05, ** p<0.001, ***p<0.001.

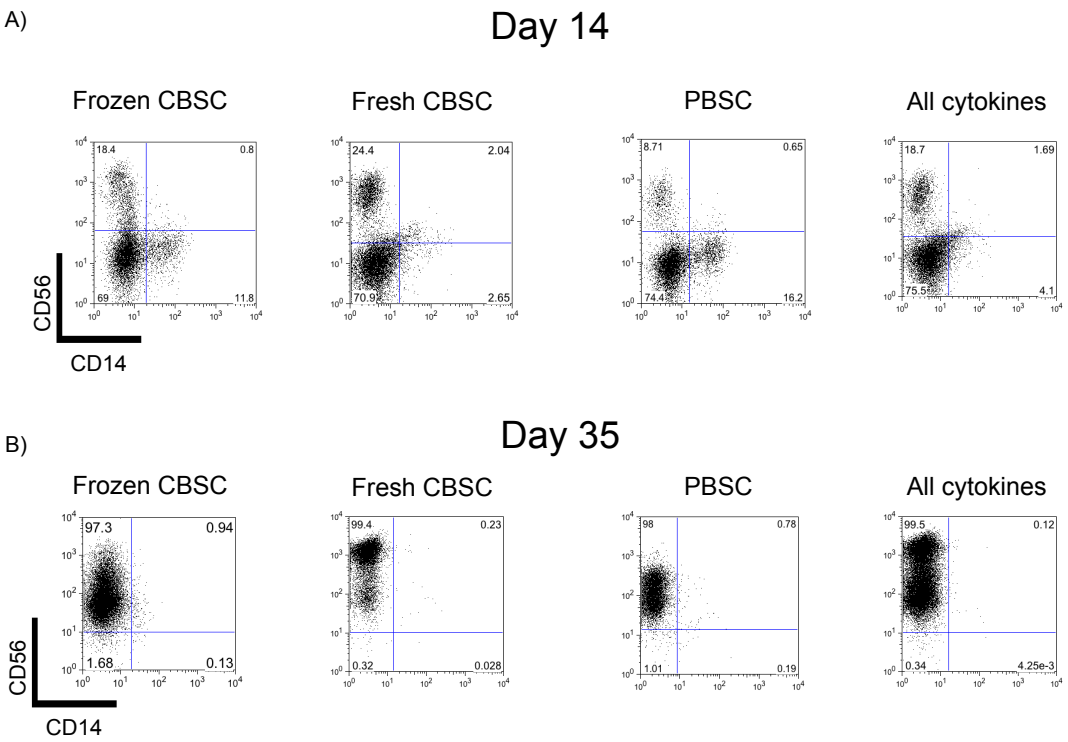


Figure 3.9. CD14 expression at days 14 and 35 in HSC cultures. Flow cytometric analysis of a representative sample for fresh CBSC (n=3), frozen CBSC (n=9), PBSC (n=6) and CBSC cultures using all cytokines (n=3). FACS plots presented come from the lymphocyte gate, cells were labelled with anti-CD56 and anti-CD14 at day 14 (A) and day 35 (B).

In summary, although a transitory expression of CD14 was observed in some cultures, the absence of monocytes was confirmed during culture of HSCs irrespectively of the source of HSC or of the cytokine cocktail used.

3.2.4 Analysis of lymphoid lineage marker expression

3.2.4.1 CD10 expression

HSC can give rise to all blood cell lineages. NK cells have been traditionally thought to originate from the lymphoid lineage; nevertheless, recent reports suggest that NK cells can also be generated from myeloid precursors (Perez *et al.*, 2003; Grzywacz *et al.*, 2011). Haddad *et al*. have shown that the expression of CD45RA, CD34 and CD7 commits cells to the T/NK cell lineage whereas the expression of CD45RA, CD34 and CD10 commits cells to the B cell lineage (Haddad *et al.*, 2004). The presence of CD45RA on CB $CD34^+$ cells was analysed; however, no $CD34^+CD45RA^+$ cells were detected (data not shown). Therefore, in this study the expression of CD45 and gated on $CD45^+CD7^+/CD10^+$ cells from the lymphocyte gate was analysed. Figure 3.10A shows the expression of CD10 in lymphocytes during the complete culture, resembling a U-shape. CD10 expression was high during the first weeks of culture, which then decreased at day 14 and 21 and finally a slight increase in CD10 expression was noted at days 28 and 35. The same tendency was found in fresh CBSC cultures using all cytokines or only IL-15 (figure 3.10B). We did not find any significant differences between cultures. It was interesting to observe the expression of CD10 at day 35 on the generated cells *in vitro*, when almost 100% of the cells are NK cells.

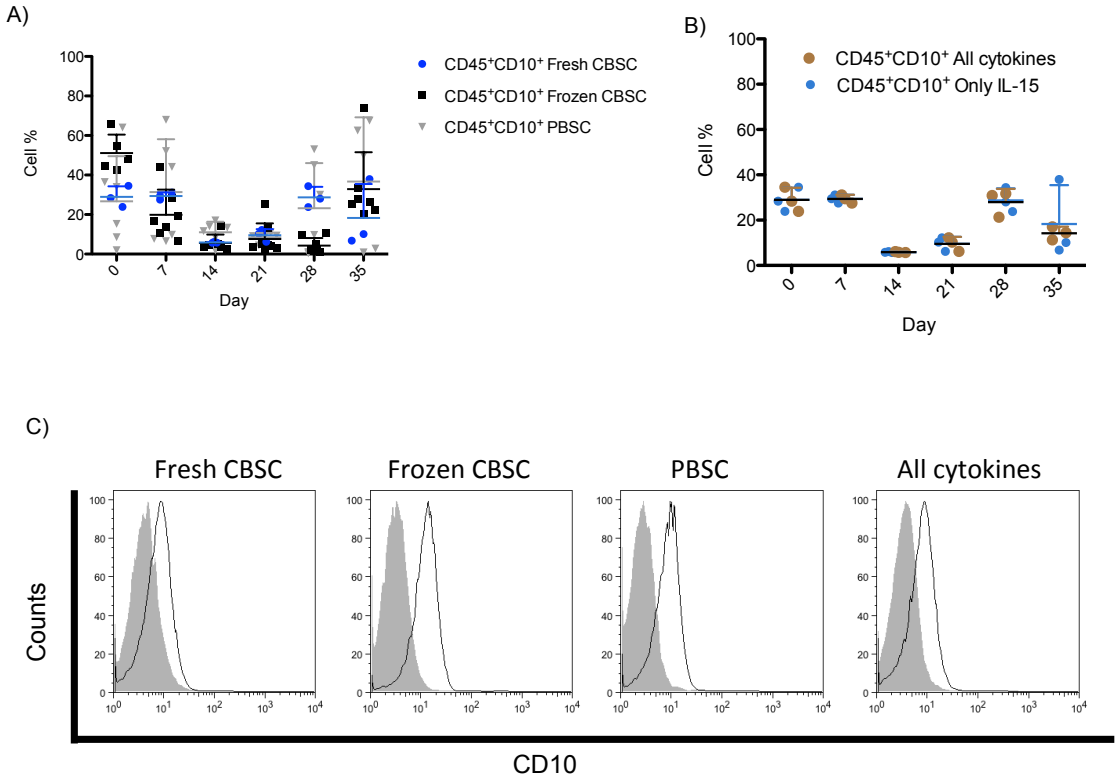


Figure 3.10. CD10 expression in HSC cultures. The percentage of $CD45^{+}CD10^{+}$ positive cells from the lymphocyte gate are presented; cells were labelled with anti-CD45 and CD10 at different time points for A) fresh CBSC (n=3), frozen CBSC (n=9), PBSC (n=6) cultures and B) CBSC cultures using all cytokines (n=3) or only IL-15 (n=3). C) Histograms showing the expression of CD10 (black line) and the control (non-stained, grey solid line) of a representative sample for fresh CBSC, frozen CBSC, PBSC and CBSC cultures using all cytokines at day 35.

Although CD10 expression has been described to define lymphoid precursors that give rise to B cells, we observed a substantial expression of CD10 on the initial HSC population and on NK cells generated *in vitro*. However, whether this population could or not differentiate into NK cells under our culture conditions remains unknown.

3.2.4.2 CD7 expression

Another lymphoid marker used to define the T/NK cell lineage together with the expression of CD45RA and CD34 is CD7 (Haddad *et al.*, 2004). The CD7 expression was analysed in the different types of culture. Surprisingly, a similar U-shape expression pattern was observed, where CD7 expression was higher at the beginning and at the end of the culture. Additionally, at the end of the culture $CD45^{+}$ cells from fresh CBSC cultures expressed more CD7 compared to frozen CBSC cultures ($p<0.05$), and $CD45^{+}$ cells from frozen CBSC expressed more CD7 than PBSC cultures ($p<0.05$) (figure 3.11A). Figure 3.11B shows that CD7 expression was high among fresh samples using all cytokines or only IL-15, but no significant difference between these cultures was found.

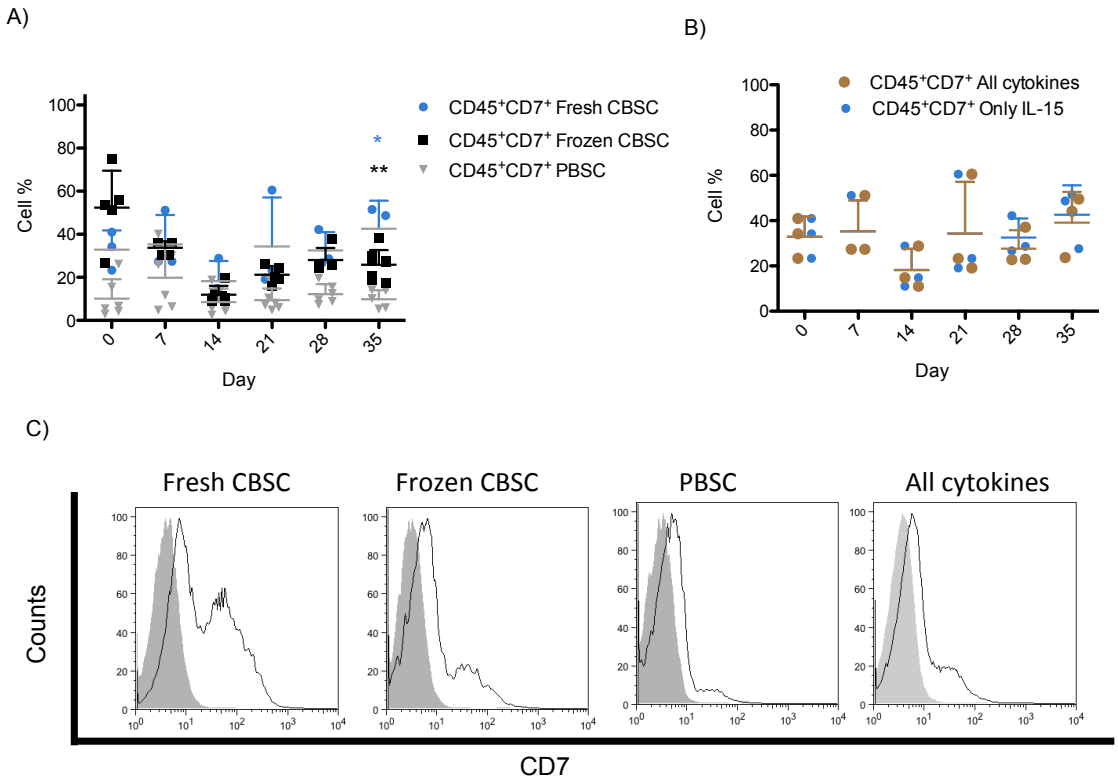


Figure 3.11. CD7 expression in HSC cultures. The percentage of $CD45^{+}CD7^{+}$ positive cells from the lymphocyte gate are presented; cells were labelled with anti-CD45 and CD7 at different time points for A) fresh CBSC (n=3), frozen CBSC (n=9), PBSC (n=6) and B) CBSC cultures using all cytokines (n=3) or only IL-15 (n=3). C) Histograms showing the expression of CD7 (black line) and the control (non-stained, grey solid line) of a representative sample for fresh CBSC, frozen CBSC, PBSC and CBSC cultures using all cytokines at day 35. Mann-Whitney test was performed, * $p<0.05$, ** $p<0.01$.

Although not all HSCs were positive for CD7, it could be noted, especially for frozen CBSC cultures, that a high percentage of $CD45^{+}CD7^{+}$ cells was found. This could partly explain the higher number of NK cells obtained in frozen CBSC cultures, however the recruitment of other precursors is a scenario that cannot be excluded in this system.

3.2.4.3 CD5 expression

T-cell development occurs when T-cell precursors migrate to the thymus where they can proliferate and subsequently give rise to mature T-cells (Blom and Spits, 2006). Early T-cell progenitors express CD5, its expression increases as the cell matures and acquisition of CD3 occurs (Weiss *et al.*, 1987). CD5 is also expressed by a subset of B

cells (Kaplan *et al.*, 2001) and has been suggested to promote B-cell survival (Gary-Gouy *et al.*, 2002). As CD5 is expressed in early lymphoid progenitors, CD5 expression was followed during the culture of HSCs (figure 3.12A and B). Surprisingly, PBSC cultures had a trend for a higher expression during the whole culture (day 28, $p<0.05$). CD5 expression among $CD45^+$ cells gradually decreased during the culture, especially in fresh CBSC cultures using all cytokines or only IL-15 (figure 3.12B).

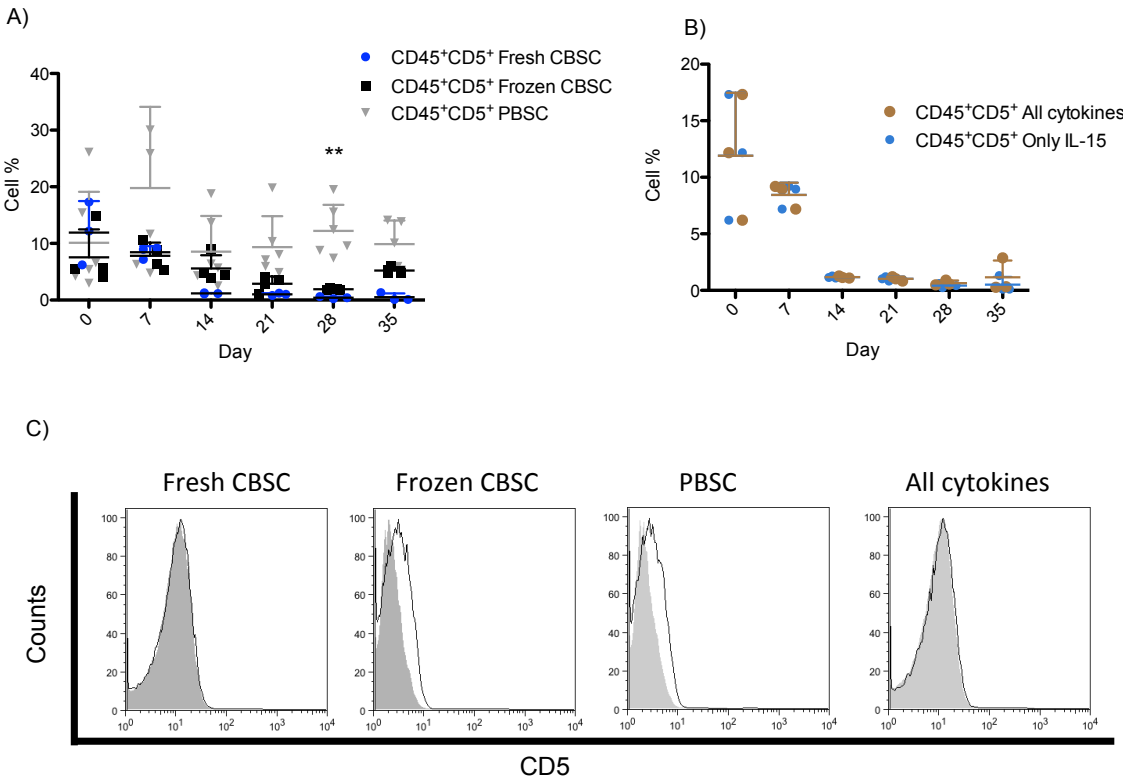


Figure 3.12. CD5 expression in HSC cultures. The percentage of $CD45^+CD5^+$ positive cells from the lymphocyte gate are presented; cells were labelled with anti-CD45 and CD5 at different time points for A) fresh CBSC (n=3), frozen CBSC (n=9), PBSC (n=6) and B) CBSC cultures using all cytokines (n=3) or only IL-15 (n=3). C) Histograms showing the expression of CD5 (black line) and the control (non-stained, grey solid line) of a representative sample for fresh CBSC, frozen CBSC, PBSC and CBSC cultures using all cytokines at day 35. Mann-Whitney test was performed. ** $p<0.01$.

As expected, CD5 expression was observed during the first weeks of HSC cultures, where lymphoid progenitors might be present; thereafter its expression decreased although PBSC cultures had higher expression of CD5 compared to CBSC cultures.

3.2.5 Analysis of myeloid lineage marker expression

Recently, there have been several works highlighting the possibility of deriving NK cells from myeloid progenitors (Perez *et al.*, 2003; Grzywacz *et al.*, 2011); as a consequence the expression of CD33, a myeloid marker, was analysed in the cultures

(figure 3.13). A high percentage of $CD45^{+}CD33^{+}$ cells throughout the culture of frozen samples was found (CBSC and PBSC). CD33 expression decreased in fresh CBSC cultures at day 21; nonetheless, values at day 35 were similar for all cultures.

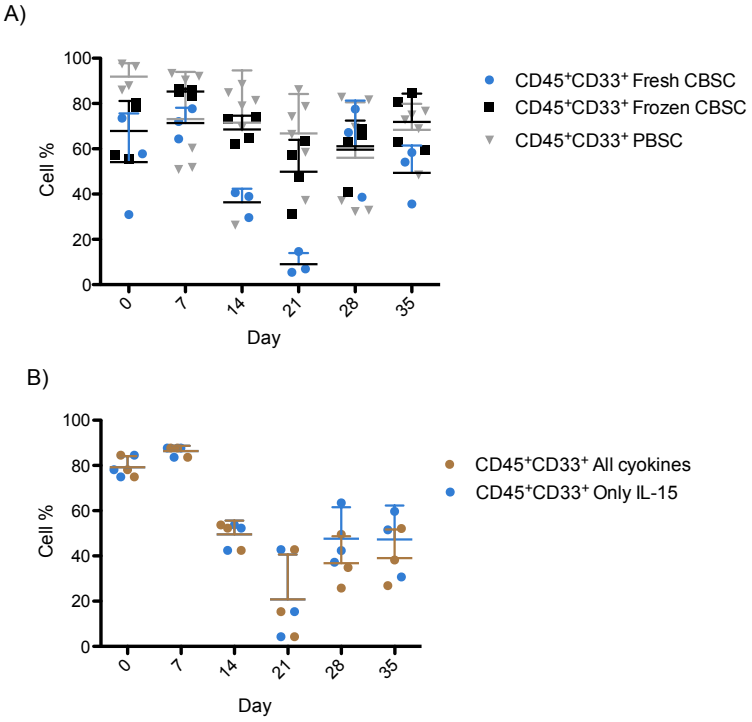


Figure 3.13. CD33 expression in HSC cultures. The percentage of $CD45^{+}CD33^{+}$ positive cells from the lymphocyte gate are presented; cCells were labelled with anti-CD45 and CD33 at different time points for A) fresh CBSC (n=3), frozen CBSC (n=9), PBSC (n=6) cultures and B) CBSC cultures using all cytokines (n=3) or only IL-15 (n=3).

Due to the high CD33 expression observed, it was investigated whether NK cells were co-expressing this marker. The expression of CD33 versus CD56 was monitored from day 7 to day 35 (figure 3.14). From these data it was possible to observe the following:

1) By day 35, almost the majority of $CD56^{bright}$ cells in fresh cultures express CD33 whereas only a fraction of $CD56^{dim}$ do.
2) In fresh cultures, $CD33^{+}$ cells at day 7 switch to the $CD56^{+}CD33^{-}$ quadrant at day 14 and 21, and later acquire CD33.
3) $CD33^{+}$ cells from frozen CBSC cultures gradually acquire CD56, whereas we can observe two distinct populations ($CD33^{+}CD56^{-}$ and $CD33^{-/low}$ $CD56^{+}$) at day 21 and 28 in PBSC cultures.

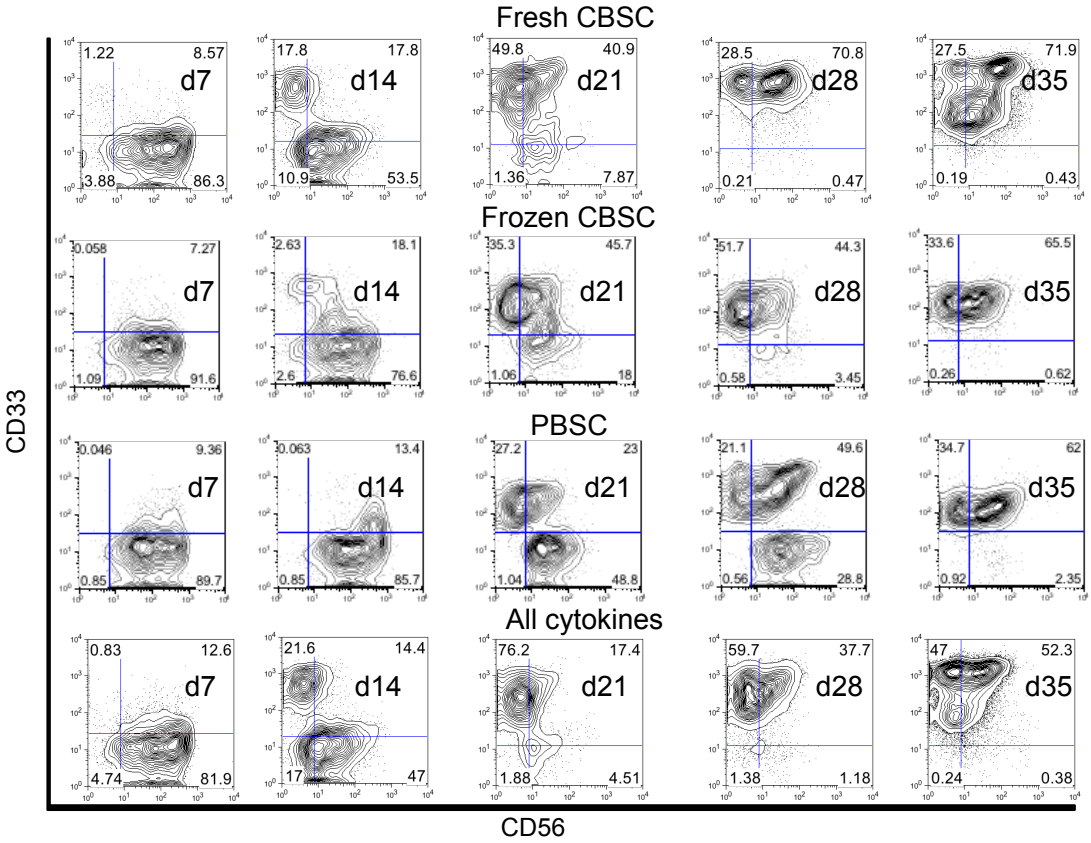


Figure 3.14. CD33+CD56+ co-expression in HSC cultures. A representative flow cytometric analysis of the expression of CD56 versus CD33 from lymphocyte gate on samples from fresh CBSC, frozen CBSC, PBSC and CBSC cultures using all cytokines at different time points is shown.

The expression of CD33 on NK cells was an unexpected observation; this myeloid marker is usually absent in PB and CB NK cells. Thus, further experiments regarding its potential role on NK cell functional features were performed and are described in chapter 6.

Altogether, the data demonstrated the presence of lymphoid and myeloid markers in HSC cultures at different time points with singular kinetics depending on the marker.

3.2.6 Natural Killer cell development in haematopoietic stem cell cultures

NK cell differentiation *in vitro* offers the opportunity to study NK cell development and numerous *in vitro* studies contributed to the identification of the factors and cytokines that drive NK cell ontogenesis *in vitro* (Miller *et al.*, 1994; Miller *et al.*, 1998; Miller and McCullar, 2001; Briard *et al.*, 2002; Grzywacz *et al.*, 2006; Beziat *et al.*, 2010; Grzywacz *et al.*, 2011; Lee *et al.*, 2011). The presence of NK cell precursors has been reported in different organs/tissues (Sanchez *et al.*, 1994; Vacca *et al.*, 2011) and the culture of these precursors has led to different concepts of NK cell development. The NK cell development model described by Freud and Caligiuri (Freud *et al.*, 2006)

identifies four NK cell stages based on the expression of the surface markers CD117, CD94 and CD34. Stage 1 is defined by the expression of $CD34^{+}CD117^{-}CD94^{-}$, stage 2 by $CD34^{+}CD117^{+}CD94^{-}$, stage 3 by $CD34^{-}CD117^{+}CD94^{-}$ and stage 4 by $CD34^{-}CD117^{+/-}CD94^{+}$. The frequency of these four stages was assessed in fresh CBSC (using all cytokines or only IL-15), frozen CBSC and PBSC cultures.

The first objective was to assess whether the use of only IL-15 for the last two weeks of culture impacted on NK cell maturation. A representative graph showing the different stages according to time for fresh CBSC cultures using all cytokines or the modified protocol is shown in figure 3.15A and 15D, respectively. No significant difference among the distribution of NK cell stages throughout the culture was observed between the two cultures (figure 3.15-17). Moreover, two different stage 3 populations were found, $CD117^{+}CD94^{-}$ and $CD117^{bright}CD94^{-}$, in fresh CBSC cultures when using all cytokines (figure 3.17). NK cell development is orchestrated by molecular events that regulate the transition from one stage to another (Hesslein and Lanier, 2011; Martin-Fontecha *et al.*, 2011; Luevano *et al.*, 2012c). TFs are key for this molecular regulation (Bezman *et al.*, 2012b; Pinho *et al.*, 2012). The expression of different TFs controlling NK cell development such as ID2, E4BP4 and RORC (figure 3.18) was investigated. The expression of E4BP4 and RORC was higher during the first two weeks of culture, whereas ID2 showed slightly higher expression during the last two weeks (figure 3.18A). These factors were expressed similarly in both types of culture without any significant difference. These results suggest that using IL-15 for the last two weeks instead of all factors did not impact negatively on NK cell development.

Next, it was analysed whether the use of frozen HSC would impact on NK cell development and maturation. A representative graph showing the different stages according to time for fresh and frozen CBSC cultures is shown in figure 3.15A and 15B, respectively. No significant differences were observed when comparing percentages of NK cell stage 1 between fresh and frozen CBSC cultures at day 0. In accordance with previous publications, a high expression of CD117 on CBSC was found (Rappold *et al.*, 1997; Wisniewski *et al.*, 2011); hence most of the cells at that time point were in stage 2. The differentiation of fresh CBSC into NK cells appeared slower for the first 2 weeks of culture, evidenced by the presence of the 4 stages by week 2 in frozen CBSC cultures (figure 3.15B). By week 3, stage 2 was almost undetectable in both cultures (figure 3.16). Stages 3 and 4 represented the majority of cells during the last two weeks of culture and their distribution was the same for both cultures (figure 3.15 and 17). Interestingly, the majority of the cells in stage 4 in fresh CBSC cultures were $CD117^{+}$, compared to cells in stage 4 in frozen CBSC cultures, which were $CD117^{low/-}$

(figure 3.17). In addition, analysis of the messenger expression for the transcription factors controlling NK cell development was performed for E4BP4, ID2 and RORC (figure 3.18). A higher expression of the these factors during the first three weeks in fresh CBSC cultures was found while the expression of these TFs was very similar in both cultures during the last two weeks of culture. In summary, frozen CBSC developed into NK cells faster, with a different expression of TFs for the first weeks of culture and a final NK cell distribution similar to that of fresh CBSC cultures with however a more mature $CD117^{low/-}$ population.

Finally, the analysis of NK cell development using two different HSC sources was performed: frozen CBSC and PBSC. As mentioned before, CBSC expressed a high level of CD117 (Rappold *et al.*, 1997), and a high incidence of stage 2 was therefore detected at day 0 (figure 3.15B) whereas both stage 1 and 2 were found in PBSC cultures at day 0 (figure 3.15C). A higher incidence of stage 3 in CBSC cultures by week 2 compared to PBSC cultures was observed, probably due to the high initial levels of stage 2 in these cultures (figure 3.17). Unlike CBSC, NK cell development was slower in PBSC cultures; however, the final distribution of stages 3 and 4 was similar at the end of the culture (figure 3.17). Moreover, we explored the expression of some TFs involved in NK cell differentiation (E4BP4, ID2, PU.1, RORC and TOX) and maturation (BCL11B, EOMES, GATA-3, HELIOS, IRF2 and T-BET) in the generated NK cells (figures 3.19 and 20). For both cultures, PU.1 was expressed more during the first 3 weeks of culture with a subsequent decrease in expression (figure 3.19), whereas E4BP4, ID2, RORC, TOX, BCL11B, EOMES, T-BET were expressed more during the last 2 weeks of culture (figures 3.19 and 20). There was a constant expression of IRF2 and a variable expression of GATA-3 during the whole culture and interestingly, HELIOS had a remarkably high expression in cells from CBSC cultures at week 1, but not in PBSC cultures (figure 3.20). The expression of some TFs was also analysed in resting CB and PB NK cells and compared to NK cells generated *in vitro* (day 35). Figure 3.21 shows that the expression of E4BP4 and HELIOS was similar among all the samples. However, ID2 was expressed more in NK cells generated *in vitro* (CBSC and PBSC, $p<0.05$) whereas a trend for a lower expression of T-BET was observed (CBSC and PBSC, $p=0.075$) compared to CB and PB NK cells. Altogether, the data suggest that PBSC cultures showed a delayed NK cell development but expressed similar levels of TFs compared to CBSC cultures.

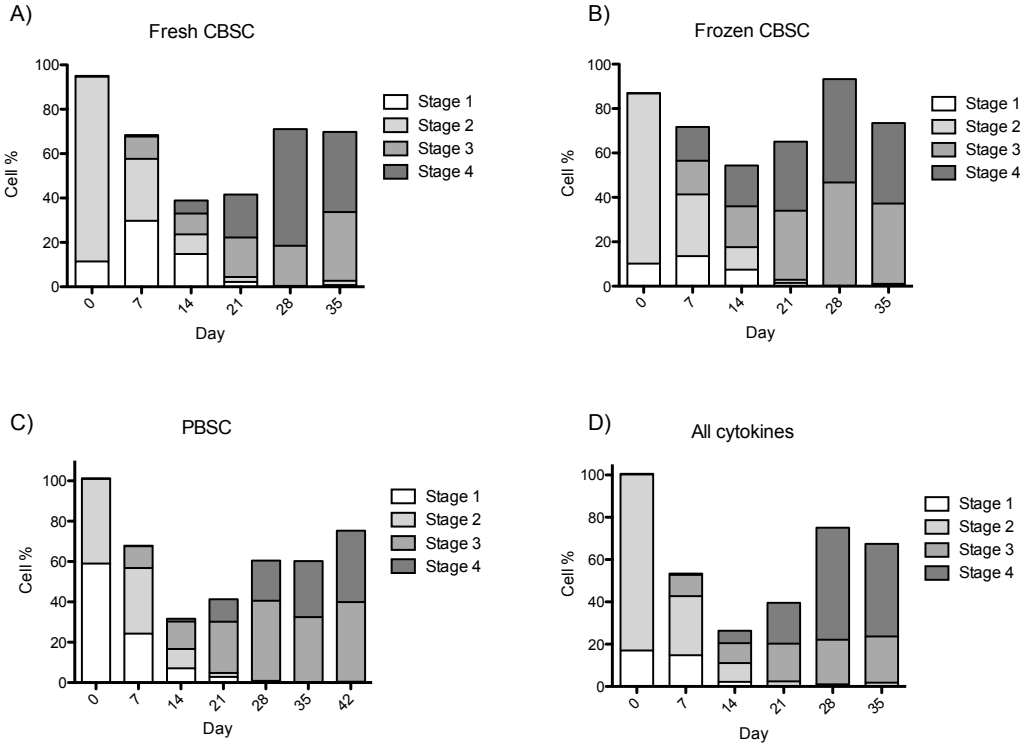


Figure 3.15. NK cell precursors in HSC cultures. Percentages represent $CD3^-$ cells from the lymphocyte gate according to the NK cell stages: stage 1: $CD34^+CD117^-CD94^-$, stage 2: $CD34^+CD117^+CD94^-$, stage 3: $CD34^-CD117^+CD94^-$ and stage 4: $CD34^-CD117^{+/-}CD94^+$. The graph shows one representative experiment for fresh CBSC (A, n=3), frozen CBSC (B, n=9), PBSC (C, n=6) and CBSC cultures using all cytokines (D, n=3) where stages 1-4 are plotted according to different time points.

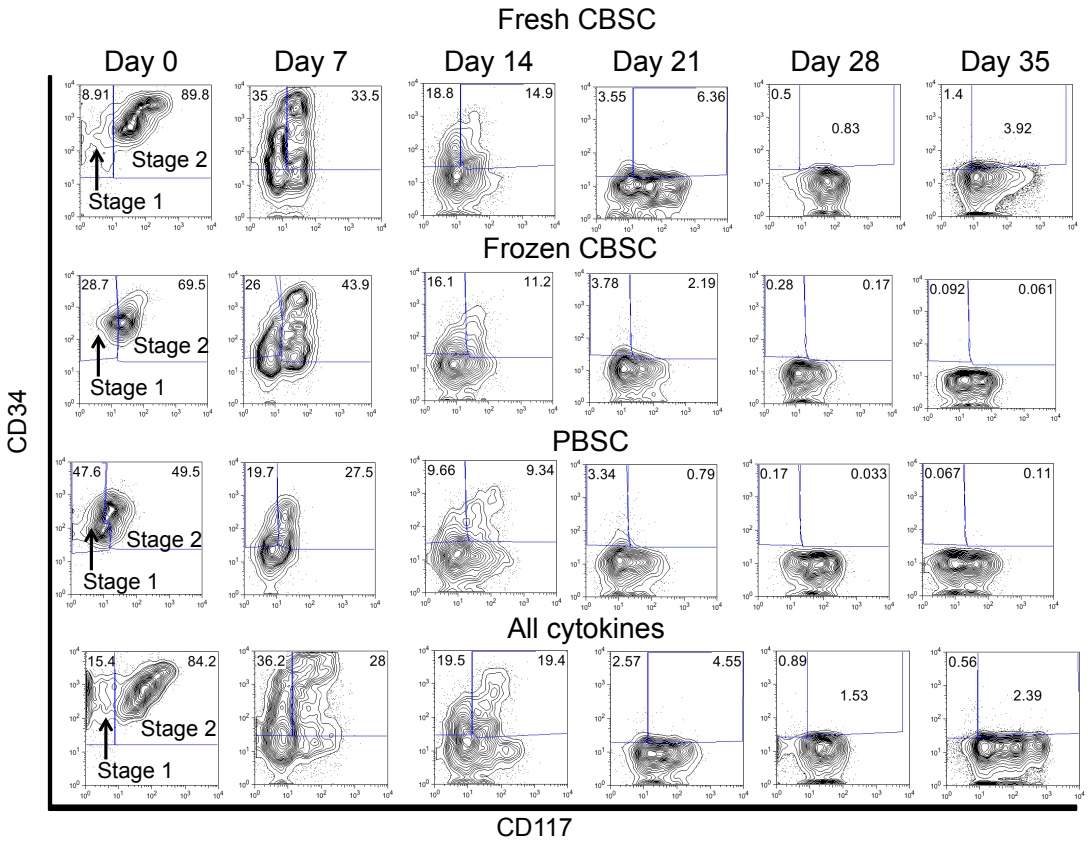


Figure 3.16. Flow cytometric analysis of NK cell stages 1 and 2 in HSC cultures. Flow cytometric analysis showing a representative sample of fresh CBSC (n=3), frozen CBSC (n=9), PBSC (n=6) and CBSC cultures using all cytokines (n=3). FACS plots showing CD34 versus CD117 expression from the $CD3^{-}CD94^{-}$ gate according to NK cell stages: stage 1: $CD34^{+}CD117^{-}CD94^{-}$, stage 2: $CD34^{+}CD117^{+}CD94^{-}$.

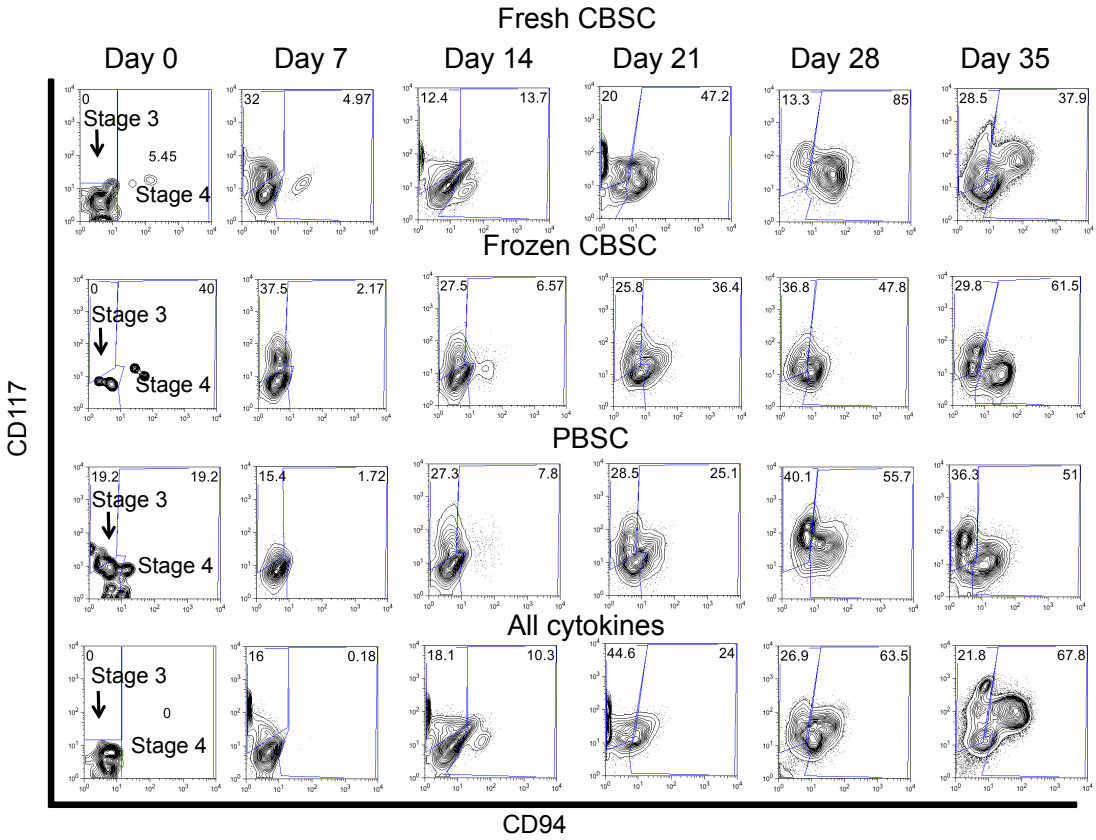


Figure 3.17. Flow cytometric analysis of NK cell stages 3 and 4 in HSC cultures. Flow cytometric analysis showing a representative sample of fresh CBSC (n=3), frozen CBSC (n=9), PBSC (n=6) and CBSC cultures using all cytokines (n=3). FACS plots showing CD117 versus CD94 expression from the $CD3^{-}CD34^{-}$ gate according to NK cell stages: stage 3: $CD34^{-}CD117^{+}CD94^{-}$ and stage 4: $CD34^{-}CD117^{+/-}CD94^{+}$.

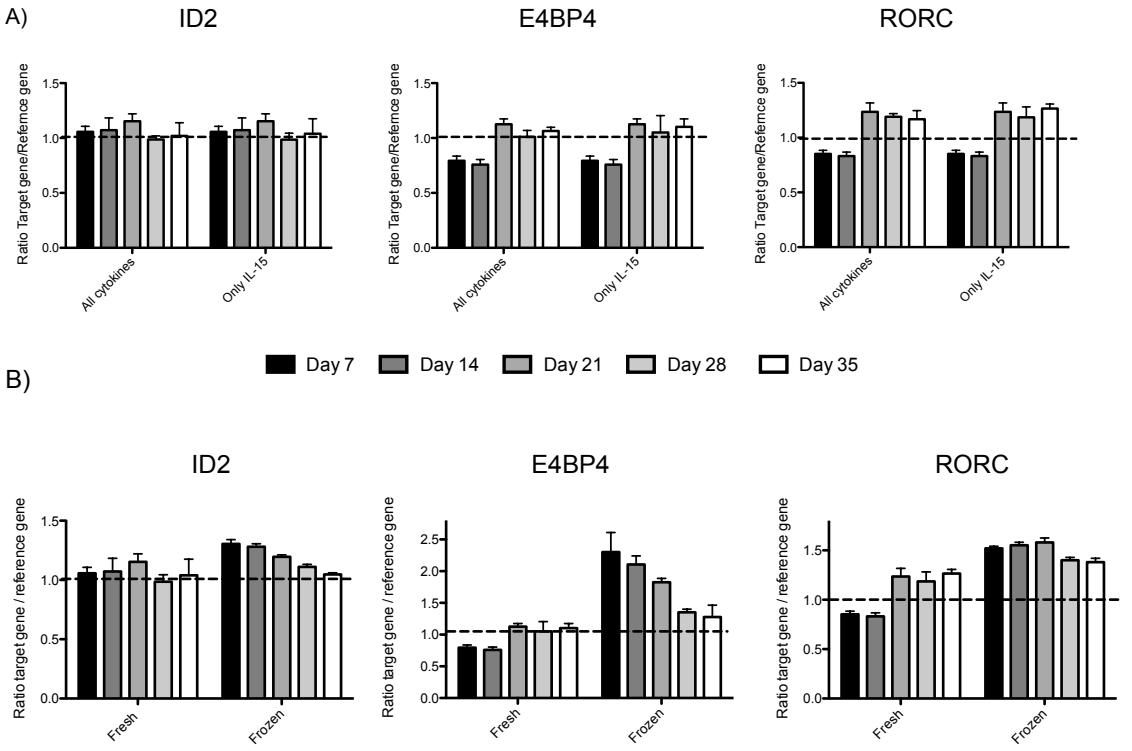


Figure 3.18. Transcription factors involved in NK cell differentiation in fresh CBSC cultures. Total RNA was extracted from fresh CBSC cultures using A) all cytokines (n=3) or B) only IL-15 (n=3) and analysed by qPCR. The mRNA expression of ID2, E4BP4 and RORC was measured at different time points. The mean and SD of the ratio target gene/reference gene is presented for each sample.

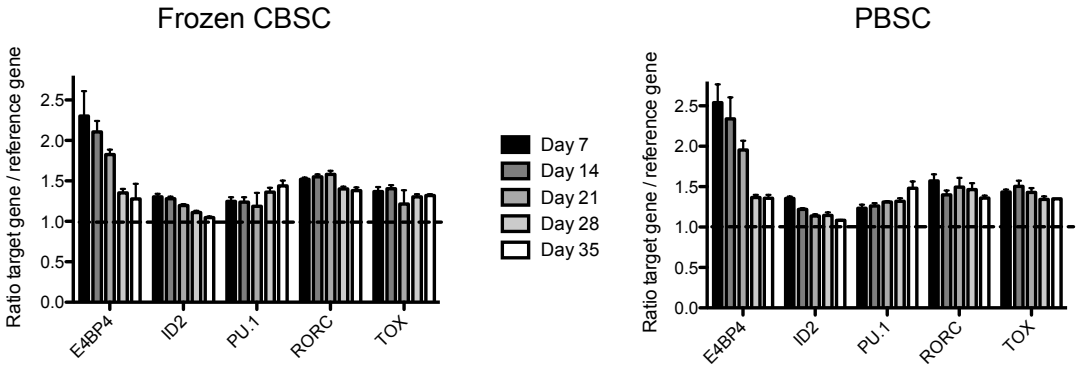


Figure 3.19. Transcription factors involved in NK cell differentiation in frozen HSC cultures. Total RNA was extracted from frozen CBSC (n=4) and PBSC (n=3) cultures and analysed by qPCR. The mRNA expression of E4BP4, ID2, PU.1, RORC and TOX was measured at different time points. The mean and SD of the ratio target gene/reference gene is presented for each sample.

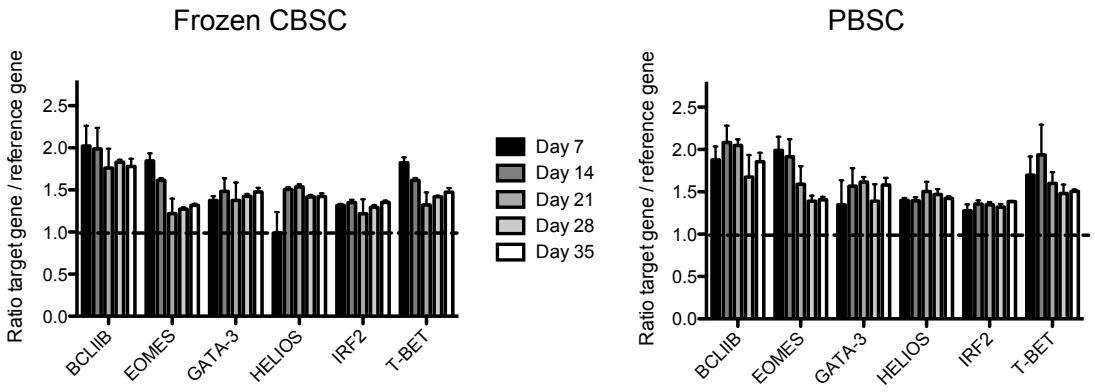


Figure 3.20. Transcription factors involved in NK cell maturation in frozen HSC cultures. Total RNA was extracted from frozen CBSC (n=4) and PBSC (n=3) cultures and analysed by qPCR. The mRNA expression of BCL11B, EOMES, GATA-3, HELIOS, IRF2 and T-BET was measured at different time points. The mean and SD of the ratio target gene/reference gene is presented for each sample**.**

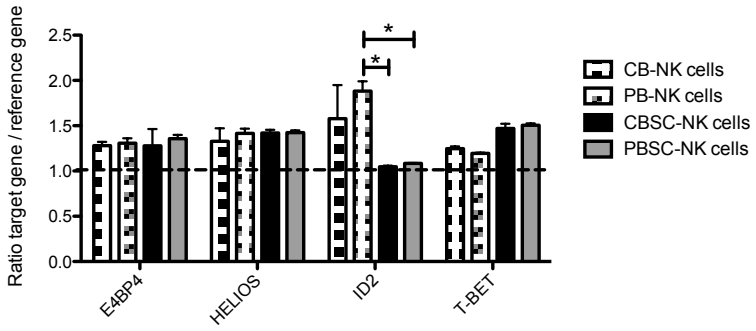


Figure 3.21. Expression of transcription factors in CB, PB, frozen CBSC and PBSC NK cells. Total RNA was extracted from resting NK cells negatively isolated from CB (n=4) and PB (n=3) and NK cells from frozen CBSC (n=4) and PBSC (n=3) cultures and analysed by qPCR. The mRNA expression of E4BP4, HELIOS, ID2 and T-BET was measured at day 35 for HSC cultures. The mean and SD of the ratio target gene/reference gene is presented for each sample. Mann-Whitney test was performed, * $p<0.05$.

3.3 Discussion

In this chapter the potential of frozen CBSC to give rise to NK cells *in vitro* was investigated and different parameters between the use of fresh and frozen CBSC, and frozen CBSC and PBSC for this approach were compared. Among these parameters the impact of using only IL-15 for the last two weeks of culture instead of all cytokines used in a published protocol was studied (Grzywacz *et al.*, 2006). Moreover, the cell expansion, viability, markers associated with different cell lineages and finally NK cell development among all cultures was studied.

The use of frozen cells is one of the most important variables for the clinical translation of a NK cell expansion protocol. Clinically, it is easier to utilise frozen cells, as they are often available off-the-shelf and can be preserved for long periods of time. The majority of the published protocols aimed at generating NK cells *in vitro* use fresh CBSC. Therefore, fresh CBSC were used and a comparison of the use of only IL-15 during the last weeks of culture to the use of a complete cytokine mix as previously reported was performed (Grzywacz *et al.*, 2006). The use of only IL-15 for the last weeks did not impact on the total fold expansion, NK cell numbers or viability. The role of IL-15 on NK cell development, proliferation, cytotoxicity and viability has been previously studied (Carson *et al.*, 1997; Cooper *et al.*, 2002; Becknell and Caligiuri, 2005). It was expected that the use of a higher IL-15 concentration for the last weeks would impact on cell expansion and viability, but it did not. One possibility would be that the IL-15 concentration used (20 ng/mL) saturated the IL-15 receptors promoting good viability and proliferation, suggesting that higher IL-15 concentration (50 ng/mL) would then not impact on these parameters.

Next, the use of frozen CBSC and fresh CBSC in terms of expansion, NK cell numbers and viability was studied. All cultures showed high viability in comparison to other works (Yoon *et al.*), and intermediate fold expansions of reported values were observed (Mrozek *et al.*, 1996; Grzywacz *et al.*, 2006). Frozen CBSC cultures showed higher and faster cell expansion than fresh CBSC cultures. This observation differs from published works where expansion of frozen HSC was often poor (Boissel *et al.*, 2008a; Boissel *et al.*, 2008b) but agreed with another study that showed frozen CBSC could be used to generate NK cells (Spanholtz *et al.*, 2010). In addition, higher NK cell numbers were retrieved using frozen CBSC compared to fresh CBSC. It is important to note that, during this work, separate CB samples were used, however, standard deviations from both cultures were low. A major difference in fold expansion between the cultures was observed from day 14 to 21, just before the withdrawal of all cytokines

and IL-15 maintenance. This suggests that the cytokine cocktail does not account for the differences in NK cell numbers and fold expansion. It is plausible that the starting population in fresh and frozen CBSC is different. Fresh CBSC may contain a combination of different HSC/progenitors; the freezing process might eliminate some of these cells leaving a population that is more viable, the 'fittest', leading to higher HSC expansion.

From that point on, all analyses were performed using frozen CBSC and the modified protocol and a comparison to another HSC source, PBSC, was performed. The data indicate that CBSC generate higher NK cell numbers compared to PBSC, while total fold expansion and viability were similar. There are only a few reports using PBSC, for instance, Zamai *et al*. observed that cells cultured using IL-15 and IL-21 had a predisposition for apoptosis, whereas the withdrawal of IL-21 gave rise to immature cells that exhibited longer survival (Zamai *et al.*, 2012). It is difficult to make a direct comparison among studies due to the use of different HSC sources and cytokine/feeder layer combinations. Though, it is interesting that Miller *et al*. observed different proliferation according to the HSC source (Miller and McCullar, 2001) and others have suggested that different hematopoietic precursors are present according to the $CD34^+$ source (BM, PB and CB) (Sconocchia *et al.*, 2004). Similar to the results in this study, Miller *et al*. reported that proliferation was greater using HSC earlier in ontogeny (foetal liver>UCB>BM) (Miller and McCullar, 2001).

All cultures showed high NK cell yield, above 90%. While any difference in NK cell yield among fresh CBSC cultures (using all cytokines or only IL-15) and frozen CBSC cultures was observed, a slower CD56 acquisition in PBSC cultures was found. Regardless of this slower CD56 acquisition, the final yield remained similar compared to CBSC cultures. Overall, the cytometric analysis showed that the generated NK cells were all $CD56^+$ but with a distinct distribution. Fresh CBSC cultures generated NK cells with two distinct populations, resembling $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets. In contrast, the majority of the frozen CBSC derived NK cells appeared to be $CD56^{dim}$, while PBSC-NK cells had no defined distribution. Confirming these findings, the CD56 MFI analysis suggested that fresh CBSC-NK cells had a tendency for higher MFI compared to frozen CBSC and PBSC. The mechanisms responsible for these differences remain unknown in the model used in this study. The impact of this CD56 distribution will have an effect on NK cell phenotype and functions, as described in the next chapters.

We also analysed the different cultures for the presence of cells that could potentially be a health risk when infused in an allogenic setting: T cells and B cells. Purity is one of the major concerns in today's clinical protocols. A recent NK cell expansion protocol by Sutlu *et al*. received criticism for having a final product with low purity (Berg and Childs, 2010; Sutlu *et al.*, 2010). As for *in vitro* generation of NK cells using HSC, the protocols in most instances favour the generation of NK cells preventing the emergence of other cell types (Spanholtz *et al.*; Miller *et al.*, 1992). In this study, a transitory expression of CD14 especially in frozen cultures (CBSC and PBSC) was observed. CD14 is expressed mainly by monocytes and macrophages (Simmons *et al.*, 1989). There is no evidence to support the lack of CD14 expression during early stages of fresh CBSC cultures. Nonetheless, CD14 expression was transitory in frozen HSC cultures and very low levels were detected at the end of the culture. In fact, none of the generated NK cells expressed CD14, supporting the absence of monocyte-like NK cells in the present study. None of the cultures exhibited significant levels of CD3 or CD19 cells, which was a great advantage as the final product was more than 90% pure, negating the need for additional positive or negative selection to eliminate unwanted cells. This has an impact on cost and also NK cell retrieval by avoiding unnecessary cell loss.

A better understanding of NK cell ontogeny has been gained in recent years. Miller *et al*. identified a $CD34^+CD7^+$ NK cell precursor using human primitive marrow progenitors (Miller *et al.*, 1994). Similarly, Haddad and colleagues described lymphoid precursors in CB (Haddad *et al.*, 2004) as characterised by the expression of CD34, CD45RA and either CD7 (T/NK-cell lineage) or CD10 (B-cell lineage). In this study, it was not possible to identify these populations (according to Haddad's study) due to the absence of CD45RA expression in the isolated CBSC. It was previously reported that CBSC have low expression of this marker (Fritsch *et al.*, 1993) at around 20%. Therefore, it was decided to gate from the lymphocyte gate on the $CD45^+$ cells. When the percentage of $CD45^+CD7^+$ and $CD45^+CD10^+$ for both conditions were analysed, interesting differences in CD7 expression were found. Initially, CD7 expression on $CD34^+$ cells had a tendency to be higher in frozen CBSC, than on fresh CBSC and PBSC cultures, which is possibly because the amount of committed NK cell progenitors was lower in PBSC. Although it has been previously described that resting NK cells have high expression of CD7 (Rabinowich *et al.*, 1994), CD7 expression remained low throughout the complete culture in PBSC cultures. In addition, CD7 has been proposed as a marker for discrimination between NK cells from monocyte/DC-like cells; with the coexpression of CD7 and CD56 defining degranulating and IFN-γ secreting cells (Milush *et al.*, 2009). By day 35, a higher CD7 expression on fresh CBSC cultures was

observed (fresh CBSC> frozen CBSC> PBSC). It is difficult to predict if NK cells will have less IFN-γ secretion or would be less able to degranulate based on CD7 expression as this suggestion would conflict with evidence advocating that low CD7 expression found on the generated NK cells can be caused by cell activation (Rabinowich *et al.*, 1994). NK cells in the culture from this study have been exposed to IL-15 for prolonged periods, providing them with an activated phenotype (discussed in the next chapter). Conversely, while studying other lymphoid lineage markers interesting data suggesting co-expression of CD10 and CD56 was found. To our knowledge, the co-expression of CD10 and CD56 on NK cells from healthy individuals has not been reported. CD10 is mainly expressed in germinal centre B cells, lymphoid progenitors, granulocytes and some T cells (Knapp *et al.*, 1989). The report of an Acute Lymphoblastic Leukaemia case co-expressing B-precursor-cell and NK-cell antigens is intriguing (Matsubara *et al.*, 2004), suggesting that NK cells generated *in vitro* differ from this biological system. Moreover, a small percentage of cells (PBSC> frozen CBSC> fresh CBSC) expressed CD5, a marker for T cells and for a subset of B cells (Kaplan *et al.*, 2001). A previous study in patients with pulmonary tuberculosis showed the presence of $CD5^{low}$ NK cells in low frequency but with high lytic activity (Ishiyama *et al.*, 1993). If the same cells also expressed CD5 and CD10 along with CD56 remains unknown in the present study, as this staining was not performed. Therefore, the impact of CD10 and CD5 expression on NK cells would need to be investigated further.

Several reports have suggested that NK cells can be derived from the myeloid lineage (Perez *et al.*, 2003; Grzywacz *et al.*, 2011) but not exclusively. In agreement with results of these studies, a recent report found that CB NK cell progenitors express CD33 from stage 2 to 3 (Eissens *et al.*, 2012). Interestingly, NK cell development data reveals that even though stage 2 cells are not present in the cultures by week 3, stage 3 cells were observed throughout the culture. It is possible that the presence of CD33 in the final product was attributed to stage 3 cells (figure 3.22). However, these observations conflict with other studies. For instance, Montaldo *et al*. also showed that $CD161^{-}CD56^{-}LFA1^{+}CD33^{+}$ cells could give rise to $CD161^{+}CD56^{+}LFA1^{-}NKp44^{+}(CD33^{-})$ (Montaldo *et al.*, 2012). During this work, $CD45^{+}CD33^{+}$ cells also give rise to $CD56^{+}$ cells. However, more than half of the generated NK cells co-expressed CD33 at day 35. In our hands, fresh CBSC cultures generated $CD56^{bright}$ cells showing more CD33 expression compared to the $CD56^{dim}$ subset. A recent report has shown that culture of G-CSF-mobilised $CD34^{+}$ using IL-2 and SCF produces $CD56^{dim}CD33^{+}$ cells and a $CD56^{bright}CD33^{+}$population. The presence of CD33 on $CD56^{bright}$ cells was attributed to the presence of IL-2 (Sconocchia *et al.*, 2004). In line with these findings, others

suggest that CD33 expression is present due to the environmental conditions in the culture (Kalberer *et al.*, 2003); furthermore, Hernandez-Caselles described that activated NK cells express an isoform of CD33 (Hernandez-Caselles *et al.*, 2006).

In summary, there are several studies revealing the role of CD33 on NK cells, but they are inconsistent and contradictory. These reports suggest that CD33 is expressed in immature cells, $CD56^{dim}$ cells or activated cells, making it difficult to conclude the function of this receptor on the generated NK cells. Figure 3.22 shows a plausible scenario combining developmental stages, CD56 expression and CD33 expression. During this study, only discrete populations in fresh CBSC cultures were found, where CD33 expression is favoured in the $CD56^{bright}$ subset (stage 4). However in theory, CD33 should be expressed only in stages 2 and 3 but since only stage 3 is present at day 35, it is possible that $CD33^{+}$ cells are actually in stage 3. The loss of CD33 might represent a maturation step that gives rise to stage 4 and 5 cells. Additional experiments were performed, and are described in chapter 6, that attempt to elucidate the role of CD33 in the cells generated in our cultures.

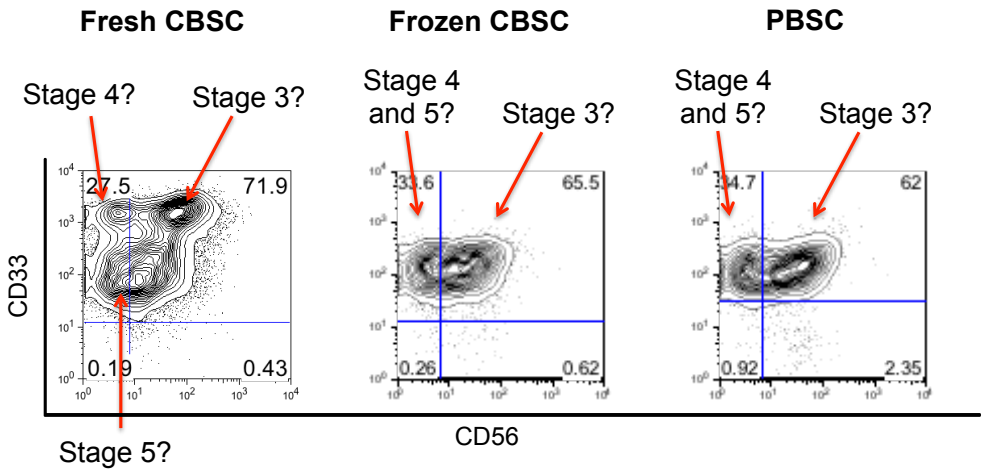


Figure 3.22. Expression of CD33 on different subsets of the generated NK cells. The figure shows the FACS plots of CD33 versus CD56 from the lymphocyte gate of NK cells derived from fresh CBSC cultures (left panel), frozen CBSC cultures (middle panel) and PBSC cultures (right panel).

NK cell development was investigated from fresh CBSC using all cytokines or only IL-15 or from frozen CBSC and PBSC. Understanding NK cell development and maturation may have implications for cancer therapy and for immunity against infections. NK cell development analysis in our study was based on the model proposed by Freud *et al*. (Freud *et al.*, 2006). This study was performed using lymph nodes (LN) precursors, thus our results might not strictly resemble those by Freud *et*

al., as our HSC sources are different. While analysing the final cell product in fresh CBSC culture using either all cytokines or only IL-15 we did not find any significant differences among the final NK cell stage distribution. Nevertheless, two different stage 3 populations were found by cytometric analysis in CBSC cultures when using all cytokines ($CD117^{+}CD94^{-}$ and $CD177^{high}CD94^{-}$). So far no differences have been found between the original protocol and the modified protocol; even the mRNA of the studied TFs remained similar between cultures. Grzywacz *et al*. described the coordinated acquisition of NK cell markers and defined stages using CD94 and CD117 receptors (Grzywacz *et al.*, 2006). In fact, this study reports the presence of intermediary subsets between $CD117^{+}CD94^{-}$ and $CD117^{-}CD94^{+}$: $CD177^{high}CD94^{-}$. Indeed, the presence of this subset only in fresh CBSC using all cytokines was observed, which was the same cytokine cocktail and cell source used in this report. We cannot exclude the existence of this subset in the fresh CBSC cultures using only IL-15 as we did not perform a daily screening. In summary, the system used supports the notion that factors like IL-7, SCF and FLT3L along with those secreted by the feeder layer are only needed for NK cell lineage commitment. Subsequently, IL-15 drives NK cell maturation as previously reported (McCullar *et al.*, 2008).

After establishing NK cell development was not affected by removal of all factors and IL-15 maintenance, we investigated whether the use of frozen CBSC would impact on NK cell development. Fresh and frozen CBSC cultures resulted in a heterogeneous population with both immature $CD117^{+}CD94^{-}$ and mature $CD117^{-/low}CD94^{+}$ NK cells, whereas other reports showed above 90% of cells in stage 4 by day 35 (Grzywacz *et al.*, 2006; Dezell *et al.*, 2012). This suggests that the transition from stage 3 to stage 4 might take longer in our culture system or may need additional signals that were not provided. When two transcription factors involved in NK cell maturation were analysed, E4BP4 and ID2, a high expression of E4BP4 during the first two weeks of culture only in fresh CBSC cultures was found. This is interesting due to the fact that E4BP4 contributes to the transition between NK cell precursors (stage 2) to iNK cells (stage 3) in previous mouse studies (Gascoyne *et al.*, 2009). Taking into account that transition from stage 2 to 3 actually took place at weeks 2 and 3 (when the highest E4BP4 expression was observed), our results correlate with the molecular profile for fresh CBSC cultures. Similarly, ID2 is essential for the transition of iNK to mNK cells (Colucci *et al.*, 2001), this TF was expressed more during the last two weeks of culture when this transition might occur. In fact, Gascoyne *et al*. suggested that ID2 is probably downstream of E4BP4 in mice (Gascoyne *et al.*, 2009). Accordingly, we observed a similar pattern in fresh HSC cultures, where E4BP4 was expressed during the first weeks and ID2 during the last two weeks.

Next, we investigated whether NK cell development in CBSC and PBSC cultures differed. Initially, PBSC cells contained a 50/50 ratio of NK cell stages 1 and 2 whereas CBSC contain mainly cells from stage 1. The impact of this different distribution at day 0 was reflected on the slower CD56 acquisition and delayed NK cell development observed in PBSC throughout the culture. When studying expression of TFs, it was found that during the first weeks of culture, only PU.1 was highly expressed, which is important for regulating the expression of stem cell factor and IL-7 receptors that play a key role in the transition of NK cell precursors to iNK and mNK (Colucci *et al.*, 2001). The data suggests that there is minimal importance of PU.1 in later stages of NK cell maturation, as previously described (Pinho *et al.*, 2012). Conversely, low expression of BCL11B in the cultures may enable expression of other TFs such E4BP4 and ID2 (Li *et al.*, 2010) that could restrict T-cell development. In line with previous reports that show that ID2 (Boos *et al.*, 2007) and TOX (Aliahmad *et al.*, 2010) are essential for the transition of iNK to mNK, these TFs were highly expressed during the last two-three weeks of the culture when this transition might occur. E4BP4 mRNA levels were similar to the one expressed by resting CB and PB NK cells. However, it was very interesting to observe that ID2 was expressed more in NK cells generated *in vitro*. A report from Pinho *et al*. found that ID2 was up-regulated during the complete culture of CB $CD34^+$ cells. This correlates with our data, suggesting that the generated NK cells are still under development and that more mature cells such as CB and PB NK cells have lower expression.

Another TF acting in the last steps of NK cell maturation is T-BET. Recent data in mice suggests that T-BET is necessary for the developmental maintenance of immature NK cells (Gordon *et al.*, 2012). Additionally, T-BET controls cytokine, perforin and granzyme B production (Townsend *et al.*, 2004; Werneck *et al.*, 2008; Jenne *et al.*, 2009; Gordon *et al.*, 2012) and, moreover, it has been reported that T-BET regulates NK cell homeostasis, promoting egress from LN and BM (Jenne *et al.*, 2009). T-BET was expressed during the last three weeks of the culture. In this study there was a tendency for CB and PB NK cells to express higher amounts of T-BET compared to generated NK cells. This is interesting, as T-BET stabilises immature NK cells and would seem plausible that generated NK cells would express more of this TF than more mature CB and PB NK cells.

A recently described TF called EOMES was reported as essential for the transition of iNK to mNK cells in mice (Gordon *et al.*, 2012) and was indeed expressed during the last three weeks of PBSC and CBSC cultures. This expression follows NK cell

maturation in our system where acquisition of cytotoxic properties occurred (chapter 5). Although controversial, it has been suggested that EOMES and T-BET control distinct checkpoints in NK cell maturation (Gordon *et al.*, 2012). T-BET stabilises the immature NK cell phenotype while EOMES allows maturation. In theory, if T-BET stabilises the immature phenotype, it should be downregulated as the cell matures, along with the increase of EOMES expression. Interestingly, CB and mature PB NK cells express higher T-BET mRNA than NK cells from CBSC and PBSC cultures. Accordingly, a suppression of T-BET when EOMES was expressed was not observed.

The maintenance of the mature NK cell pool in peripheral blood is in part provided by the expression of IRF-2, deficiency of which causes accelerated apoptosis (Taki *et al.*, 2005). Although our system does not distinguish between biological compartments, we observed a constant and low expression of IRF-2 in the generated NK cells, suggesting this factor is not directly involved in NK cell development, but could be important for NK cell survival throughout the culture. Finally, we studied GATA-3 expression, another TF that is not directly involved in NK cell commitment but known to be important for IFN-γ production in mice (Samson *et al.*, 2003; Vosshenrich *et al.*, 2006) and highly expressed in $CD56^{bright}$ NK cells in humans (Vosshenrich *et al.*, 2006). Some reports suggest that GATA-3 regulates T-BET; as T-BET regulates IFN-γ production it is possible that the effect of GATA-3 on IFN-γ production is an indirect result of GATA-3 regulating T-BET (Samson *et al.*, 2003). We hypothesised that the increasing expression of GATA-3 will correlate with T-BET expression; however a variable expression was found. The molecular data obtained during this work correlates with some of the current roles elucidated by work using mouse models, however, there are still some discrepancies. A complete screening of mRNA TFs in CB $CD34^{+}$ *in vitro* differentiation system was recently performed (Pinho *et al.*, 2012). Some of our findings differ from the ones published by Pinho *et al*., however a different system was used. Among the most interesting differences, Pinho *et al*. found a downregulation of TOX and E4BP4 and a gradual increase of GATA-3 throughout the culture (Pinho *et al.*, 2012). The authors suggested that TOX and E4BP4 may have an impact only during the first week of culture, and its involvement in later development stages is minimal. The results from this study suggest that PU.1 is the most important factor in the beginning of the culture and then other TFs play a key role as their expression emerges when the transition from iNK to mNK cells occurs. Our data does not exactly match those of published work in humans and in mice but provides a different insight into NK cell development that will be later complemented with phenotype and functionality maturation (chapters 4 and 5).

In summary, this chapter provides new evidence that frozen CBSC are a feasible source of HSC for producing NK cells with results comparable to fresh CBSC and even to PBSC. A detailed study of cell lineages in the different cultures and the study of the development of these cells provide evidence for the different behaviour of PBSC cultures. Importantly, we report here for the first time that higher NK cell numbers can be achieved using frozen CBSC rather than fresh CBSC or PBSC. If a thawed clinical grade UCB unit contains an average of 2.79 x 10^6 cells (Spanholtz *et al.*, 2011a), we could produce as many as 1.6 x 10^9 NK cells. Alternatively, isolated HSC can be aliquoted and used to produce NK cells for multiple infusions at different time points. In the next chapters the phenotype and functions of the generated NK cells are analysed in detail.

Chapter 4: Phenotype of Natural Killer cells generated *in vitro* from haematopoietic stem cells

4.1 Introduction

NK cells are promising tools for immunotherapy due to their ability to kill malignant cells without prior antigen priming. Before NK cell generation *in vitro* was considered an option for NK cell adoptive therapy, PB was the main source of NK cells. There are two NK cell subsets in human PB based on CD56 expression, $CD56^{bright}$ and $CD56^{dim}$. These subsets have a different distribution within the body, and exhibit different cell-surface receptor repertoires and functions. Traditionally, it has been shown that $CD56^{bright}$ NK cells have a predominant immunoregulatory role and $CD56^{dim}$ cells are more cytotoxic. Nonetheless, a recent report suggested that $CD56^{dim}$ NK cells are rapid producers of IFN-γ, providing immunoregulatory properties to this subset (De Maria *et al.*, 2011). Based on this, should we aim to produce NK cells *in vitro* with a $CD56^{dim}$ phenotype for immunotherapy?

For decades, isolated PB NK cells have been expanded or activated *ex-vivo* using cytokines, such as IL-2 (Torelli *et al.*, 2002). Several reports in which PB NK cells are expanded for immunotherapy perform a characterisation focusing mainly on the expression of activating and inhibitory receptors (Fujisaki *et al.*, 2009; Siegler *et al.*, 2010; Voskens *et al.*, 2010). It seems that NK cells that have been activated or manipulated for a long period of time (Escudier *et al.*, 1994; Torelli *et al.*, 2002; Alici *et al.*, 2008) are more potent compared to NK cells activated overnight or for a short period of time (Burns *et al.*, 2003; Krause *et al.*, 2004) as shown in clinical studies. So what is the phenotype of these long-term activated NK cells making them effective killers? NK cells rely on a vast combinatorial array of receptors to initiate their effector function; to mount a response NK cells must integrate signals transmitted through stimulatory and inhibitory receptors and in particular counteract the influence of inhibitory receptors. Alici *et al*. suggest that upregulation of 2B4, NCRs, NKG2D and DNAM-1 contribute to the killing of malignant melanoma cells (Alici *et al.*, 2008). Accordingly, the higher cytotoxicity of NK cells expanded in a bioreactor was attributed to the upregulation of NKp44 (Sutlu *et al.*, 2010). So, is it possible to obtain a similar phenotype for NK cells that have been generated *in vitro*?

Each protocol for NK cell generation *in vitro* will provide cells with different phenotype and activation state. Such differences in NK cell characteristics will have an impact on their efficiency in a clinical setting. As previously mentioned, different sources of HSC have been used to generate NK cells *in vitro* (Yoon *et al.*; Miller *et al.*, 1994; Mrozek *et al.*, 1996; Spanholtz *et al.*, 2010; Spanholtz *et al.*, 2011a; Dezell *et al.*, 2012; Lehmann *et al.*, 2012; Zamai *et al.*, 2012). Zamai *et al*. generated NK cells from PBSC $CD34^+$ cells that resembled $CD56^{bright}$ cells. NK cells expressed granzyme B, perforin, LFA-1, low levels of KIRs and CD16. After stimulation with IL-15, NK cells upregulated granzyme B, TRAIL, Fas-L, CD16, KIRs and LFA-1 (Zamai *et al.*, 2012). Yoon *et al*. also generated NK cells from PBSC, resulting in NK cells expressing NKp46 and NKG2D but that had very low KIRs expression (Yoon *et al.*).

CB has been used in several studies to generate NK cells. As for NK cells generated from PBSC, NK cells generated from CB mainly had a $CD56^{bright}$ NK cell phenotype. The characterisation of the generated NK cells *in vitro* shows a lack of CD16 and KIRs expression, which suggests that NK cells generated in this system were still immature (Beck *et al.*, 2009; Bonanno *et al.*, 2009). Since 2010, Spanholtz *et al*. have been developing an *in vitro* system to generate NK cells from CBSC. This system deserves particular attention, as it is one of the first to produce NK cells under GMP guidelines, and additionally has a feeder layer-free system (Spanholtz *et al.*, 2010; Spanholtz *et al.*, 2011a). Generated NK cells in this system express a variety of activating markers: NKG2A/D, 2B4, CD161, NCRs. Their phenotype resembles that of $CD56^{bright}$ NK cells, with low expression of KIRs and CD16. Accordingly, Kao *et al*. used a serum-free protocol to expand CB $CD34^+$ HSC and produce NK cells *in vitro*. Their characterisation only included perforin, Fas-L and CD16, concluding that $CD56^{bright}CD16^-$ NK cells expressing Fas-L but not perforin were generated. Some reports have shown that CB $CD34^+$ cells could be differentiated into $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets (Yu *et al.*, 2001; Sivori *et al.*, 2003) whereas others suggested that $CD34^+$ cells could only generate $CD56^{bright}$ cells *in vitro* (Mrozek *et al.*, 1996; Sconocchia *et al.*, 2005b).

As described, the majority of current protocols give rise to $CD56^{bright}$ cells *in vitro*. In most studies, the characterisation of the generated cells is limited to the expression of some activating and inhibitory receptors with few of them assessing expression levels of adhesion molecules and receptors involved in migration (Beck *et al.*, 2009; Woll *et al.*, 2009). However, this is of great importance, as NK cells need to migrate to the tumour site and proliferate sufficiently (Albertsson *et al.*, 2003). Moreover, NK cells need to express adhesion molecules to allow conjugate formation and cytolytic granule

polarisation in order to kill malignant cells effectively (Bryceson *et al.*, 2005). To our knowledge, there is currently no study performing characterisation of NK cells generated *in vitro* and including chemokine receptors, integrins, interleukin receptors, activating/inhibitory receptors and even co-stimulatory receptors (see appendix 2).

The characterisation of the NK cell repertoire would allow a better correlation between the phenotype of infused NK cells and the clinical outcome. Therefore, a full characterisation of NK cells derived from two different HSC sources and cultured under different conditions as described in the previous chapter was performed. Using flow cytometry and qPCR, the phenotypic characteristics of NK cells generated from fresh and frozen CBSC, fresh CBSC (using only IL-15 or all cytokines during culture) and NK cells produced from PBSC were assessed. After analysing the frequency of NK cell progenitors and their development in all HSC cultures, the work undertaken for this chapter will help to correlate the developmental status of NK cells generated *in vitro* together with their phenotype.

4.2 Results

4.2.1 Natural Killer cells generated *in vitro* express NKG2A but lack KIRs expression

Inhibitory receptors play a major role in protecting host cells against NK cell mediated killing. Some of these receptors act upon interaction with MHC class I molecules, whereas others bind to non-MHC class I molecules. These receptors include the inhibitory KIRs and the inhibitory NKG2A heterodimeric C-type lectin-like receptors. KIRs bind to classical MHC class Ia molecules (HLA-A, -B, and –C) while NKG2A binds to non-classical MHC class Ib (HLA-E) (Lanier, 2001; Lanier, 2008). The acquisition of some inhibitory receptors on the generated NK cells from all HSC cultures at day 35 was analysed. NK cells from HSC cultures were used without further purification by magnetic isolation (NK cell content in HSC cultures varied from 93-97%). Cells were detached directly from the 96-well plates by resuspension, transferred to a falcon tube to be washed and counted for subsequent use in phenotypic and functional assays. Figure 4.1 shows the expression of the inhibitory markers CD158a (KIR2DL1/DS1), CD158b (KIR2DL2/3) and NKG2A on NK cells. A very low expression of CD158a and CD158b on NK cells from each culture was observed, whereas NKG2A was expressed on 40-50% of NK cells without any significant difference between cultures.

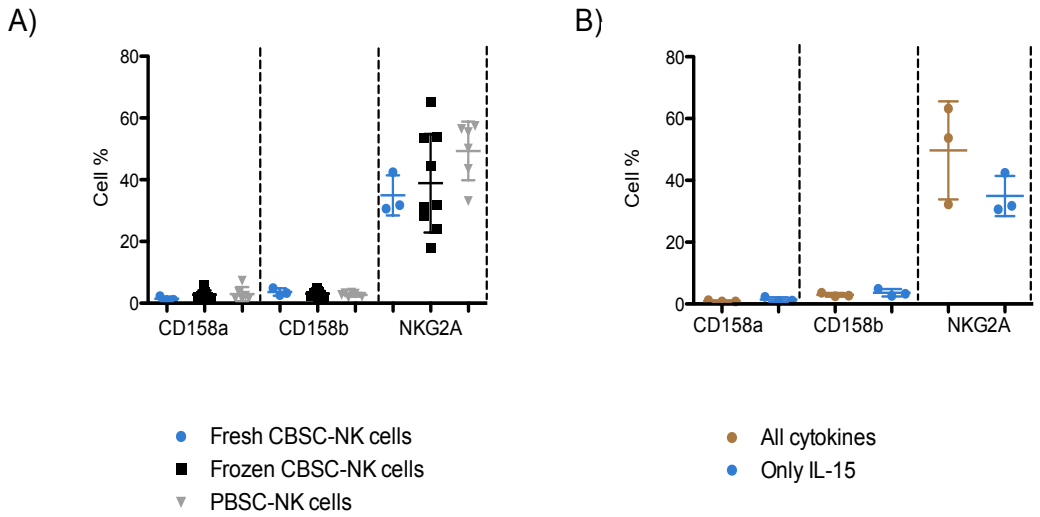


Figure 4.1. Expression of inhibitory receptors on NK cells generated in vitro. Graphs show the cell expression (mean ± SD) of CD158a, CD158b and NKG2A on the generated NK cells (percentages come from the $CD56^{+}CD3^{-}$ gate) at day 35 from A) fresh CBSC (n=3), frozen CBSC (n=9), PBSC (n=6) cultures and B) CBSC cultures using all cytokines (n=3) or only IL-15 (n=3). Mann Whitney test was performed.

Previous studies showed that as $CD56^{dim}$ NK cells mature they expressed more CD158a and CD158b and less NKG2A (Beziat *et al.*, 2010). Thus, a further analysis of

CD158a, CD158b and NKG2A in CD56^{bright} and CD56^{dim} NK cell subsets from frozen CBSC and PBSC cultures was performed (figure 4.2 A-D). CD158a and CD158b expression was significantly higher in the CD56^{dim} subset ($p<0.05$) in CBSC cultures and the same tendency was observed in PBSC cultures although it was not statistically significant ($p=0.100$). Likewise, NKG2A expression was higher in the CD56^{dim} NK cell subset (CBSC, $p<0.05$).

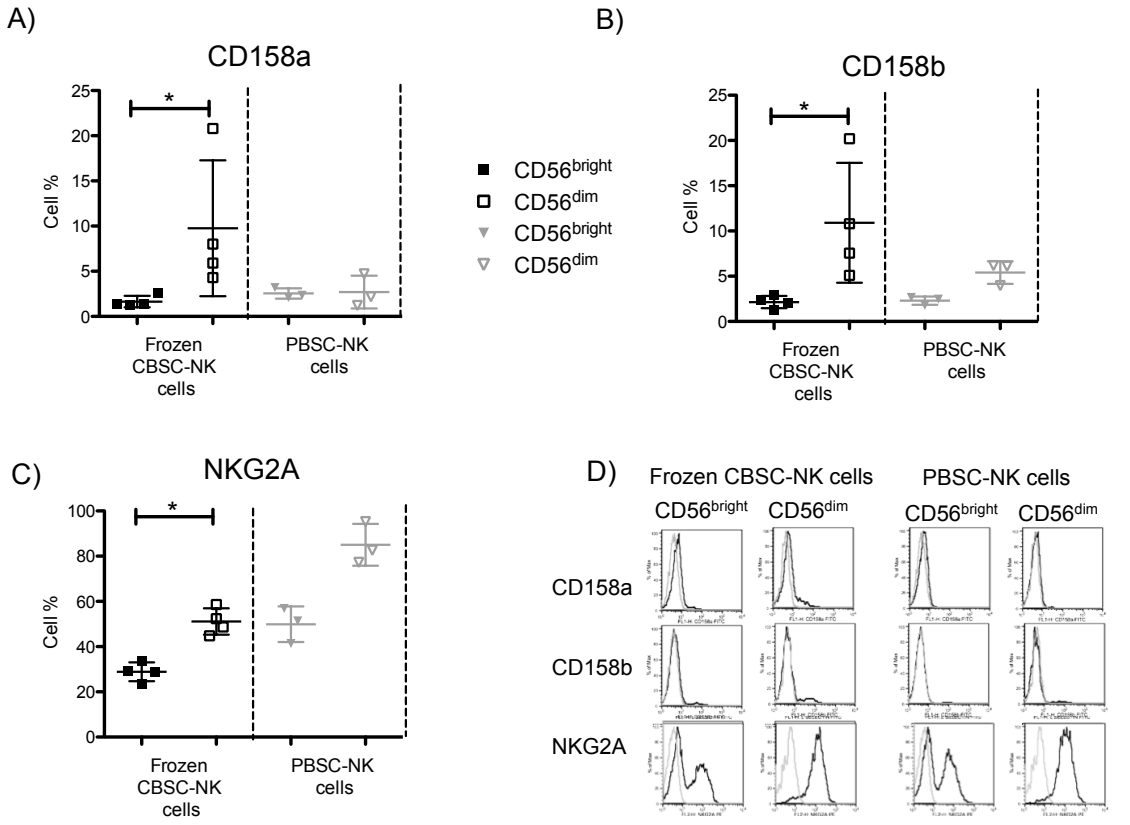


Figure 4.2. Expression of inhibitory receptors in CD56^{bright} and CD56^{dim} subsets. Graphs depict the expression at day 35 of CD158a (A), CD158b (B) and NKG2A (C) on NK cells (percentages come from the CD56^{+}CD3^{-} gate) from frozen CBSC (n=4) and PBSC (n=3) cultures. D) Figure shows the histograms of one representative sample for CD158a, CD158b and NKG2A expression in CD56^{bright} and CD56^{dim} subsets from frozen CBSC and PBSC NK cells. Mann Whitney test was performed. * $p<0.05$, ** $p<0.01$.

4.2.1.1 KIRs mRNA expression

During NK cell maturation, sequential acquisition of KIRs occurs as NKG2A progressively decreases (Beziat *et al.*, 2010). Since these markers have been associated not only with NK cell maturation but also with NK cell licensing/education process (Anfossi *et al.*, 2006; Foley *et al.*, 2011a), their absence led us to investigate their mRNA expression levels using qPCR. Figures 4.3 and 4.4 show the relative expression of these markers in NK cells from HSC cultures at day 35. Resting NK cells

from CB and PB were included as controls. Figure 4.3 depicts the expression of KIRs; KIR2DL1 expression was similar among CB, PB and fresh CBSC cultures. NK cells from cultures where all cytokines were used exhibited the highest KIR2DL1 expression while NK cells from frozen cultures had the lowest expression. The expression of KIR2DL2 remained very similar among NK cells from CB, PB and all HSC cultures.

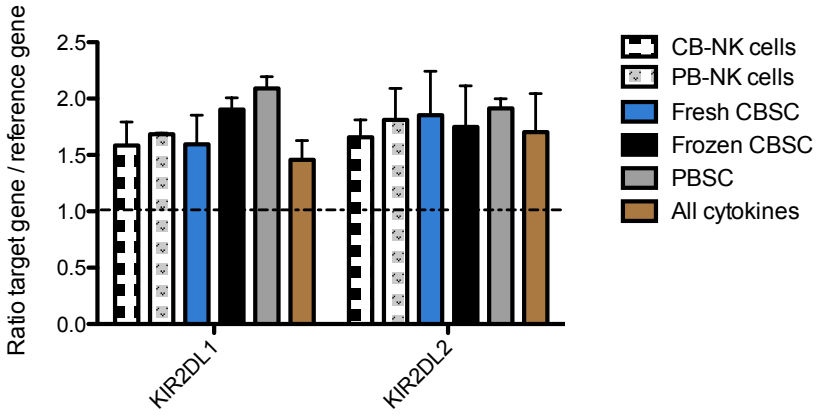


Figure 4.3. KIRs mRNA expression. Total RNA was extracted from NK cells from PB (n=3), CB (n=3), fresh CBSC cultures (n=3), frozen CBSC cultures (n=4), PBSC cultures (n=3) and CBSC cultures with all cytokines (n=3) and analysed by qPCR. Figure shows the relative expression of KIR2DL1 and KIR2DL2 in NK cells at day 35. The mean and SD of the ratio target gene/reference gene for each sample is presented.

In summary, KIRs expression in PB and CB NK cells as well as in the generated NK cells was very similar. Although the reason(s) for the low KIRs expression detected by flow cytometry are unclear, the possibility of other post-translational modifications cannot be excluded.

4.2.1.2 NKG2A mRNA expression

The analysis of NKG2A mRNA levels showed less expression in CB and PB NK cells compared to NK cells generated *in vitro*; a tendency for higher expression of NKG2A in NK cells from fresh CBSC cultures was found (figure 4.4).

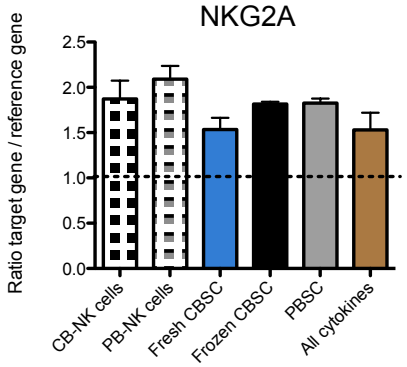


Figure 4.4. NKG2A mRNA expression in NK cells from CB, PB and HSC cultures. Total RNA was extracted from NK cells from CB (n=3), PB (n=3), fresh CBSC cultures (n=3), frozen CBSC cultures (n=4), PBSC cultures (n=3) and CBSC cultures with all cytokines (n=3) and analysed by qPCR. Figure shows the relative expression of NKG2A in NK cells at day 35. The mean and SD of the ratio target gene/reference gene for each sample is presented.

The acquisition of NK cell functions is correlated with NKG2A expression (Grzywacz *et al.*, 2006), therefore mRNA expression of this marker in frozen CBSC and PBSC cultures was analysed. Figure 4.5 shows an increase in NKG2A mRNA levels throughout the culture. In recent studies, it has been suggested that CD57 cell expression correlates with a more mature stage than $CD56^{dim}$ only, characterised by the loss of expression of NKG2A and the gain of KIR receptor expression (Bjorkstrom *et al.*; Lopez-Verges *et al.*). Considering the majority of the generated NK cells were $CD56^{bright}$, it is feasible that either the few $CD56^{dim}$ have not undergone the last step of maturation recently proposed, or that the number of cells in that stage was so low that we could not detect a decrease in NKG2A mRNA levels.

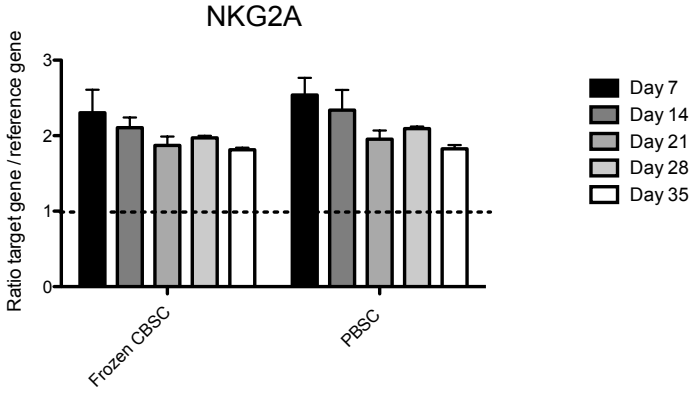


Figure 4.5. NKG2A mRNA expression at different time points of culture of CBSC and PBSC. Total RNA was extracted from NK cells from frozen CBSC cultures (n=4) and PBSC cultures (n=3) at day 7, 14, 21, 28 and 35 and analysed by qPCR. Figure shows the relative expression of NKG2A in NK cells. The mean and SD of the ratio target gene/reference gene for each sample is presented.

4.2.2 Abundant NCRs expression on the generated Natural Killer cells

NK cells express a large number of cell-surface markers that can be classified as activating or inhibitory receptors. The study of some of the inhibitory receptors was previously described. In this section, the presence of an important group of activating receptors named NCRs was investigated; NKp30, NKp44, NKp46 and NKp80. NKp30 and NKp46 are expressed on resting and activated NK cells, whereas NKp44 is only expressed on activated NK cells (Moretta *et al.*, 2000). NKp80 is expressed in fresh and activated NK cells, acting as an activating receptor (Vitale *et al.*, 2001). Figure 4.6 shows that except for NKp80, the remaining NCRs were highly expressed without any significant difference between NK cells from the different HSC cultures. It is not surprising to find high expression of NCRs on the generated NK cells, as the media used in the cultures contained IL-15, which is known to activate NK cells.

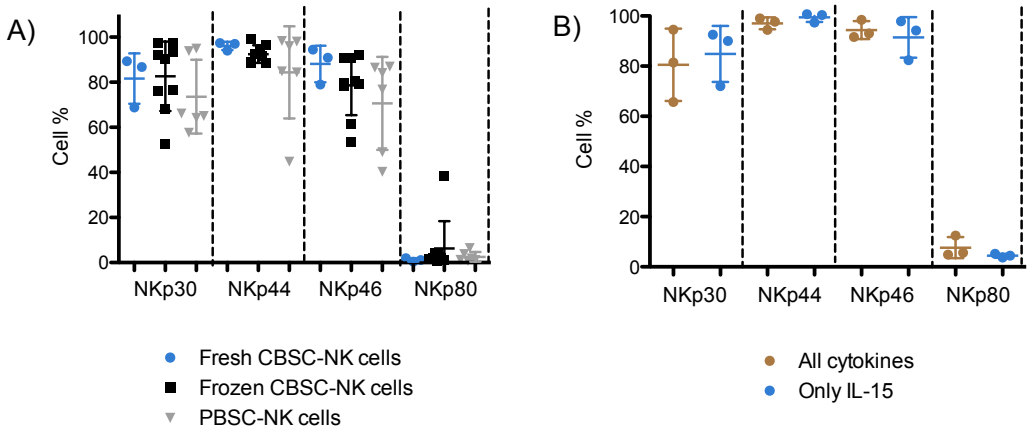


Figure 4.6. Expression of natural cytotoxic receptors on NK cells generated. The graphs show the cell expression percentage and SD of natural cytotoxic receptors (NCRs) at day 35 on NK cells (percentages come from the $CD56^{+}CD3^{-}$ gate) from A) fresh CBSC (n=3), frozen CBSC (n=9), PBSC cultures (n=6) and B) CBSC cultures with all cytokines (n=3) or only IL-15 (n=3). Mann Whitney test was performed.

While analysing the $CD56^{bright}$ and $CD56^{dim}$ NK cell populations, it was observed that only NKp46 was differentially expressed between subsets with higher expression on $CD56^{dim}$ cells (CBSC-NK cells, $p<0.05$, figure 4.7).

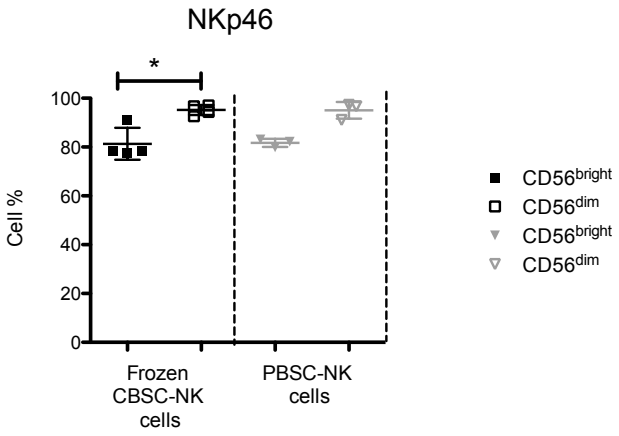


Figure 4.7. Expression of NKp46 in CD56^{bright} and CD56^{dim} NK cell subsets. Graphs depict the expression of NKp46 at day 35 on NK cells (percentages come from the CD56^{+}CD3^{-} gate) from frozen CBSC (n=4) and PBSC (n=3) cultures. Mann Whitney test was performed, * p<0.05.

4.2.3 Absence of 2B4 expression on the generated Natural Killer cells

Recent advances in NK cell biology have highlighted novel roles for different NK cell receptors, such as 2B4. This receptor has a dual function; it acts as an inhibitory receptor during early development and as the cell matures, acts as an activating receptor (Sivori *et al.*, 2002). Its ligand, CD48, is expressed on a variety of cells including NK cells; 2B4-CD48 interactions enhance NK cell proliferation in response to interleukins (Assarsson *et al.*, 2004). Figure 4.8 depicts 2B4 and CD48 expression on NK cells from all HSC cultures. 2B4 is usually expressed in 100% of NK cells however 2B4 expression was very low in NK cells from all HSC cultures (4.5-10.1%). On the contrary, CD48 was expressed on NK cells from frozen cultures (32.9 ± 28.8% and 45.8 ± 22.3%, CBSC and PBSC, respectively). Interestingly, fresh CBSC-NK cells had very low CD48 expression (6.9 ± 4.6%).

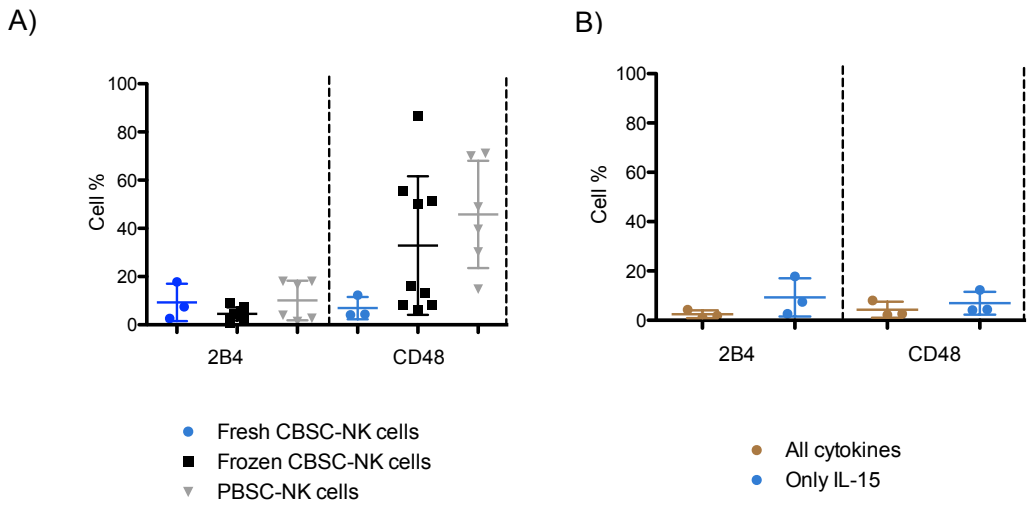


Figure 4.8. Expression of co-stimulatory receptors on NK cells. The graphs show the cell expression percentage and SD of 2B4 (CD244) and CD48 at day 35 on NK cells (percentages come from the $CD56^{+}CD3^{-}$ gate) from A) fresh CBSC (n=3), frozen CBSC (n=8), PBSC cultures (n=6) and B) CBSC cultures with all cytokines (n=3) or only IL-15 (n=3). Mann Whitney test was performed.

4.2.3.1 2B4 mRNA expression

Due to the low expression of the surface antigen 2B4 using flow cytometry, the analysis of the mRNA levels for this marker in NK cells using qPCR was performed. Figure 4.9 depicts the mRNA expression of 2B4 in CB NK cells, PB NK cells, and NK cells from all HSC cultures. mRNA levels of CBSC and PBSC samples resemble those of CB NK cells and PB NK cells respectively. This suggests that 2B4 expression in NK cells generated *in vitro* is similar to their counterparts in CB and PB.

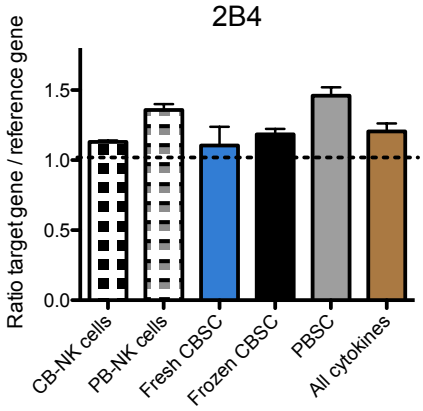


Figure 4.9. 2B4 mRNA expression in NK cells. Total RNA was extracted from NK cells from CB (n=3), PB (n=3), fresh CBSC cultures (n=3), frozen CBSC cultures (n=4), PBSC cultures (n=3) and CBSC cultures with all cytokines (n=3) and analysed by qPCR. Figure shows the relative expression of 2B4 in NK cells at day 35. The mean and SD of the ratio target gene/reference gene for each sample is presented.

Because 2B4 is an important receptor during NK cell development, its mRNA expression throughout CBSC and PBSC cultures was assessed. Figure 4.10 depicts the weekly levels of 2B4 mRNA for both HSC cultures. While a steady level was maintained in PBSC cultures, CBSC showed high expression at day 7 and 35.

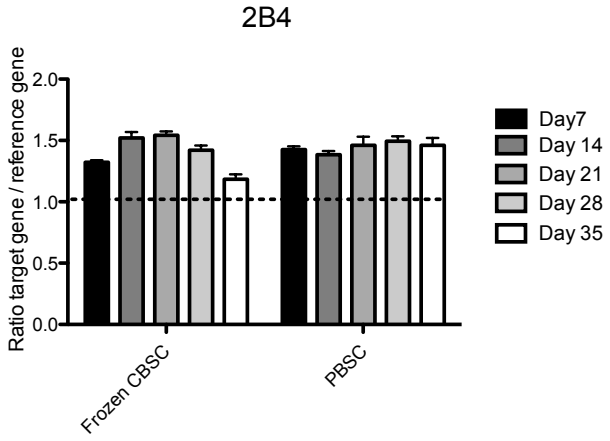


Figure 4.10. 2B4 mRNA expression at different time points of culture of CBSC and PBSC. Total RNA was extracted from NK cells from frozen CBSC cultures (n=4) and PBSC cultures (n=3) at day 7, 14, 21, 28 and 35 and analysed by qPCR. Figure shows the relative expression of 2B4 in NK cells. The mean and SD of the ratio target gene/reference gene for each sample is presented.

Altogether, regardless of the presence of 2B4 mRNA in NK cells from CBSC and PBSC cultures at similar or even higher levels than CB and PB NK cells, additional events might be occurring preventing the expression of the protein.

4.2.4 Generated Natural Killer cells are equipped with cell death-receptors and activating Natural Killer cell receptors

The two major mechanisms effecting NK cell cytotoxicity are the perforin/granzyme and the death-receptor-mediated (Fas-L or TRAIL-mediated) induction of cell death pathways (Trapani *et al.*, 1998; Wallin *et al.*, 2003). NK cells can express death receptors: Fas-L and TNF-related-apoptosis-inducing ligand (TRAIL) (Caron *et al.*, 1999; Wallin *et al.*, 2003). In this study, the receptors Fas-L and TRAIL were studied as part of the phenotypic characterisation of NK cells. Fas belongs to the TNF receptor family of molecules containing a conserved intra-cytoplasmic “death domain” that indirectly activates the caspase enzymatic cascade and ultimately induces apoptotic mechanisms in numerous cell types (Screpanti *et al.*, 2005). Fas-L expression on NK cells contributes to suppression of tumour cells *in vitro* (Oshimi *et al.*, 1996; Zamai *et al.*, 1998a). Fas-L was expressed similarly between fresh and frozen CBSC-NK cells (figure 4.11). Conversely, PBSC-NK cells had a higher Fas-L expression compared to

CBSC-NK cells (figure 4.11, p<0.05). TRAIL can bind to death receptor (DR)4 and DR5 leading to the recruitment of the protein FADD, which functions as a molecular bridge to caspase 8, a protease that initiates cell death cascade (Gibson *et al.*, 2000). NK cells use TRAIL to mediate apoptosis in some tumours (Takeda *et al.*, 2002) or immature DCs (Hayakawa *et al.*, 2004). A higher expression of TRAIL was found in frozen cultures (CBSC-NK and PBSC-NK cells) compared to fresh CBSC-NK cells (p<0.05); and between the frozen cultures CBSC-NK cells had higher expression compared to PBSC-NK cells (figure 4.11, p<0.05).

On the other hand, NK cells are equipped with activating receptors able to trigger NK cell killing. The heterodimer CD94/NKG2C also binds to HLA-E, but associates with DAP12, a membrane receptor containing an ITAM motif. NKG2D is expressed as a homodimer that associates with another adaptor molecule, DAP10. NKG2D recognises at least eight ligands on the surface of human cells, each with MHC class I homology: four transmembrane proteins, MICA and MICB, ULBP4-5, and four glycophosphatidylinositol-anchored proteins, ULBP1-3 and ULBP6 (Raulet *et al.*, 2013). These two receptors play an important role in cellular activation. NK cells generated from all HSC cultures expressed NKG2D but had very low expression of NKG2C (figure 4.11), although a high variation in frozen CBSC-NK cells was found. Thus, NKG2C mRNA levels in CB NK cells, PB NK cells and NK cells generated *in vitro* were investigated. mRNA levels in NK cells generated *in vitro* were similar to CB NK cells; PB NK cells had slightly lower expression levels (figure 4.12). NK cells from all cultures expressed NKG2D without any significant difference (figure 4.11).

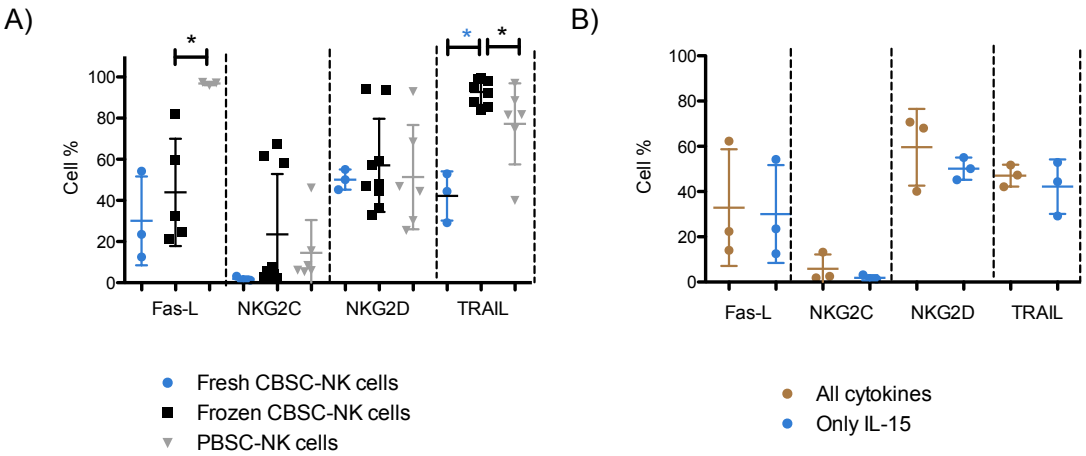


Figure 4.11. Expression of activating receptors on NK cells. The graphs show the cell expression (mean ± SD) of Fas-L, NKG2C and NKG2D at day 35 on NK (percentages come from the $CD56^{+}CD3^{-}$ gate) cells from A) fresh CBSC (n=3), frozen CBSC (n=9), PBSC cultures (n=6) and B) CBSC cultures with all cytokines (n=3) or only IL-15 (n=3). Mann Whitney test was performed, * p<0.05.

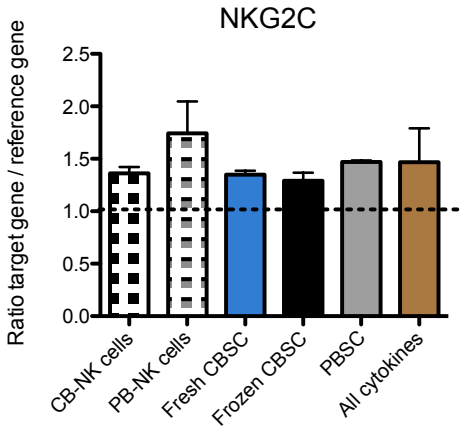


Figure 4.12. NKG2C mRNA expression in NK cells. Total RNA was extracted from NK cells from CB (n=3), PB (n=3), fresh CBSC cultures (n=3), frozen CBSC cultures (n=4), PBSC cultures (n=3) and CBSC cultures with all cytokines (n=3) and analysed by qPCR. Graph shows the relative expression of NKG2C in NK cells at day 35. The mean and SD of the ratio target gene/reference gene for each sample is presented.

Finally, we investigated the presence of one of the most important NK cell activating receptors able to trigger NK cell cytotoxicity without costimulation: CD16 (Bryceson *et al.*, 2005). The engagement of CD16 receptor facilitates ADCC against transformed/malignant cells coated with IgG antibodies. Previous studies report the generation of $CD56^{+}CD16^{low/-}$ cells *in vitro* using CBSC or PBSC (Mrozek *et al.*, 1996; Sconocchia *et al.*, 2005b). Fresh CBSC-NK cells from either all cytokines or only IL-15 cultures had a tendency for higher CD16 expression (p=0.0714) while frozen CBSC-NK and PBSC-NK cells had reduced expression (no significant difference, figure 4.13).

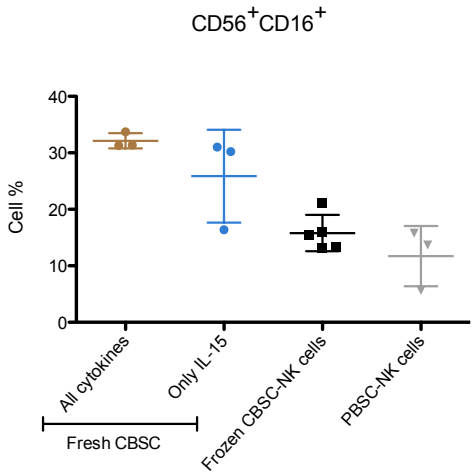


Figure 4.13. NK cells generated *in vitro* express CD16. Graph shows the percentage of $CD56^{+}CD16^{+}$ cells from the lymphocyte gate of fresh CBSC cultures with all cytokines (n=3) or only IL-15 (n=3), frozen CBSC cultures (n=5) and PBSC cultures (n=3). Mann Whitney test was performed.

In summary, NK cells generated *in vitro* had high expression of death-ligand receptors and NKG2D, and low expression of NKG2C and CD16.

4.2.5 Interleukin receptor expression profile on Natural Killer cells generated *in vitro*

NK cells can proliferate and secrete IFN-γ and other factors in response to interleukins. The study of the interleukin receptor repertoire on NK cells generated *in vitro* can be helpful for the prediction of NK cell responses when these interleukins are administered *in vivo* or *in vitro*. The expression of IL-15Rα, IL-18R, IL-2Rα, IL-2Rβ1 and IL-12Rβ1 at day 35 on the generated NK cells was investigated. From all the receptors studied during this work, the screening of interleukin receptors showed the most variable expression among cultures (figure 4.14). IL-15Rα and IL-12Rβ1 were more expressed in fresh CBSC-NK cells, while IL-2Rα and IL-2Rβ1 expression was higher in frozen CBSC-NK cells ($p<0.05$, figure 4.14). IL-18R was more expressed on PBSC-NK cells compared to frozen CBSC-NK cells ($p<0.005$). No significant differences between CBSC cultures using all cytokines or only IL-15 were found.

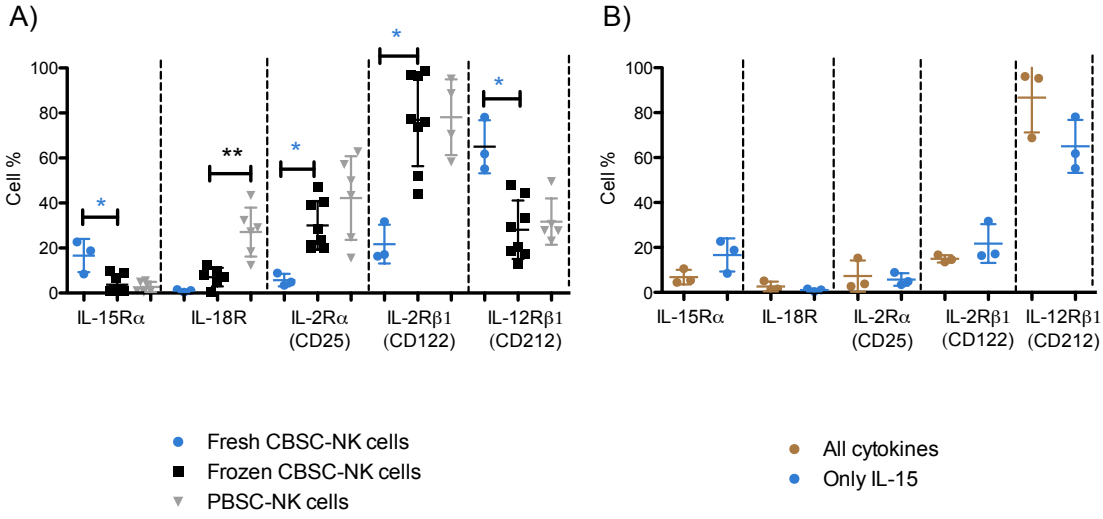


Figure 4.14. Expression of interleukin receptors on NK cells. The graphs show the cell expression (mean ± SD) of IL-15Rα, IL-18R, IL-2Rα, IL-2Rβ1 and IL-12Rβ1 at day 35 on NK cells (percentages come from the $CD56^{+}CD3^{-}$ gate) from A) fresh CBSC (n=3), frozen CBSC (n=9), PBSC cultures (n=6) and B) CBSC cultures with all cytokines (n=3) or only IL-15 (n=3). Mann Whitney test was performed, * $p<0.05$, ** $p<0.01$.

Due to the different functions of NK cell subsets (immunoregulatory or cytotoxic), interleukin receptors can be differentially expressed accordingly. Indeed, IL-12Rβ1 was more expressed in CBSC $CD56^{bright}$ NK cells (figure 4.15A). Conversely, IL-18R was more expressed in CBSC $CD56^{dim}$ NK cells (figure 4.15B). A representative analysis

using flow cytometry to analyse expression of IL-12Rβ1 and IL-18R in CBSC-NK and PBSC-NK cells is presented in figure 4.15C.

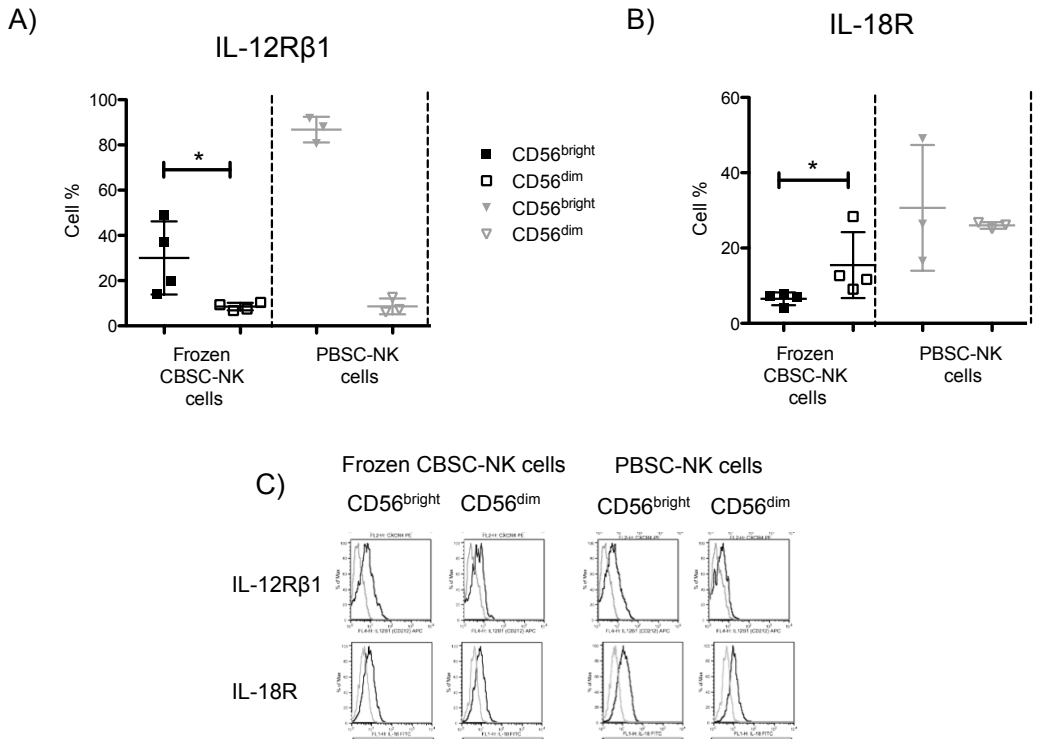


Figure 4.15. Interleukin receptor expression in CD56^{bright} and CD56^{dim} subsets. Graphs depict the expression at day 35 of IL-12Rβ1 (A) and IL-18R (B) on NK (percentages come from the CD56^{+}CD3^{-} gate) cells from frozen CBSC (n=4) and PBSC (n=3) cultures. C) Figure shows the histograms of one representative sample for IL-12Rβ1 and IL-18-R in CD56^{bright} and CD56^{dim} subsets from frozen CBSC and PBSC NK cells. Mann Whitney test was performed. * $p<0.05$.

In summary, frozen CBSC-NK cells had higher expression of CD25 and CD122 compared to fresh CBSC-NK cells. Both receptors are important for IL-2 and IL-15 signalling, suggesting that frozen CBSC-NK cells might respond better to these cytokines.

4.2.6 Frozen CBSC-NK cells generated *in vitro* express CXCR4

NK cells have been tested for immunotherapeutic properties with promising results in non-solid malignancies (Miller *et al.*, 2005) and less encouraging outcomes in solid tumours (Ishikawa *et al.*, 2004). Regardless of the nature of the tumour, NK cells must be able to make contact with malignant cells in order to mediate cytotoxicity. Characterising the trafficking potential of the generated NK cells will provide valuable

information for immunotherapeutic uses. This work included the analysis of the expression of chemokine receptors that allow cell trafficking to different organs/tissues such as CCR5 (liver), CCR6 (gut mucosa) (Hirata *et al.*), CCR7 (lymph nodes) (Campbell *et al.*, 2001), CXCR1 (inflammatory sites) (Maghazachi, 2010), CXCR4 and CXCR7 (bone marrow) (Beider *et al.*, 2003; Hartmann *et al.*, 2008). Figure 4.16 shows the expression of these chemokine receptors on the NK cells generated *in vitro*. High variability among samples was observed, especially frozen samples, whereas fresh CBSC-NK cells had a very low expression of most of the studied chemokine receptors. The only significant difference was found in the expression of CXCR4 between NK cells from fresh and frozen CBSC cultures ($p<0.05$). The use of different all cytokines or only IL-15 had no effect on the expression of the aforementioned chemokine receptors (figure 4.16B).

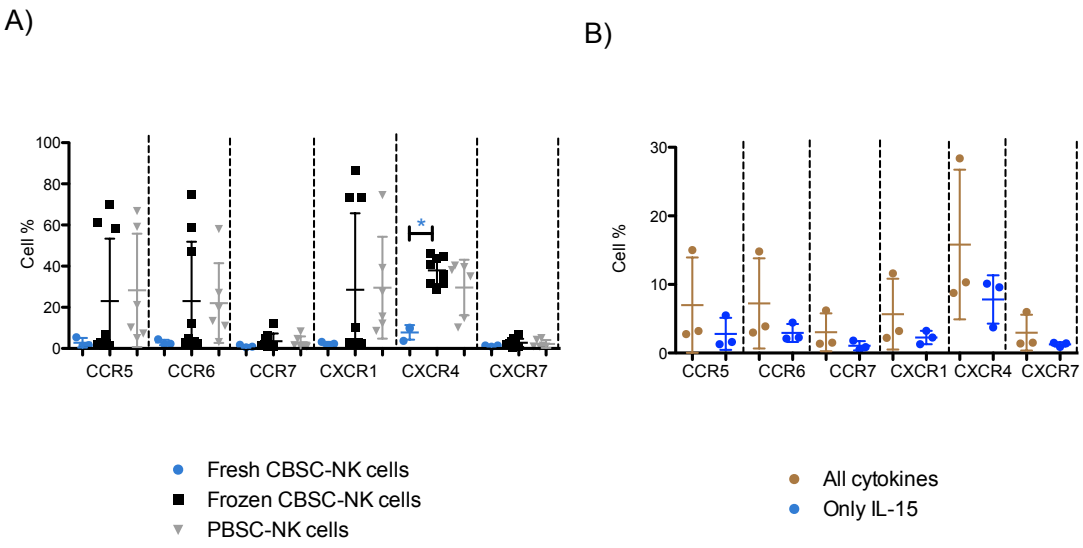


Figure 4.16. Expression of chemokine receptors on NK cells. The graphs show the cell expression (mean ± SD) of CCR5, CCR6, CCR7, CXCR1, CXCR4 and CXCR7 at day 35 on NK cells (percentages come from the $CD56^{+}CD3^{-}$ gate) from A) fresh CBSC (n=3), frozen CBSC (n=9), PBSC cultures (n=6) and B) CBSC cultures with all cytokines (n=3) or only IL-15 (n=3). Mann Whitney test was performed. * $p<0.05$.

In addition, the expression of these markers in $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets from frozen CBSC and PBSC cultures was analysed. CXCR4 expression was higher in the CBSC $CD56^{bright}$ subset (figure 4.17A). A representative sample for NK cell subsets of frozen CBSC and PBSC cultures is shown in figure 4.17B.

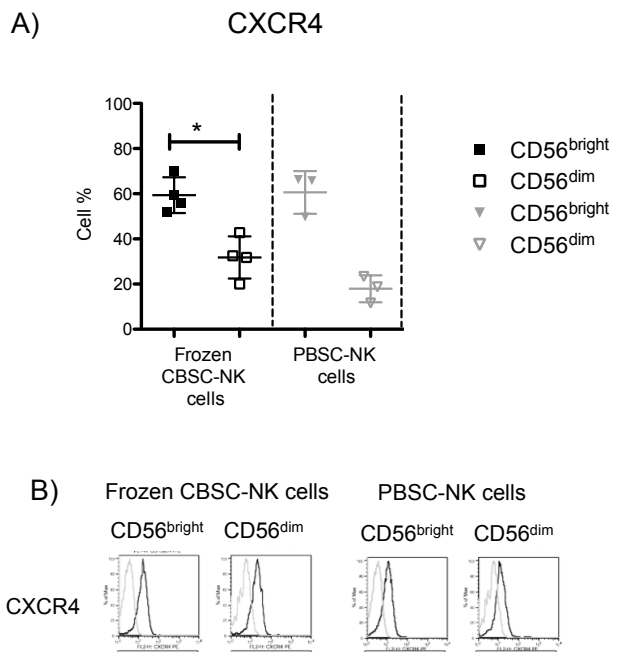


Figure 4.17. Expression of CXCR4 in CD56bright and CD56dim subsets. A) Graph depicts the expression at day 35 of CXCR4 on NK cells (percentages come from the $CD56^{+}CD3^{-}$ gate) from frozen CBSC (n=4) and PBSC (n=3) cultures. B) Figure shows the histograms of one representative sample for CXCR4 in $CD56^{bright}$ and $CD56^{dim}$ subsets from frozen CBSC and PBSC NK cells. Mann Whitney test was performed. * $p<0.05$.

Taking into consideration the importance of CXCR4 in BM homing, frozen CBSC-NK cells and PBSC-NK cells would have the potential to migrate to the BM whereas fresh CBSC-NK cells may not.

4.2.7 Natural Killer cells generated *in vitro* express adhesion molecules

Finally, the expression of integrins that allow cell adhesion and homing was studied: CD49d (transendothelial migration) (Humphries *et al.*, 1995), integrin β7 (interaction with extracellular matrix and endothelial cells) (Perez-Villar *et al.*, 1996), L-selectin (lymph node homing), DNAM-1 and LFA-1 (cell adhesion) (Tahara-Hanaoka *et al.*, 2004; Mace *et al.*, 2010). CD49d was expressed in the majority of fresh and frozen CBSC-NK cells, while its expression tended to be higher in PBSC-NK cells (although not statistically significant, figure 4.18A). NK cells from all cultures expressed DNAM-1; fresh and frozen CBSC-NK cells had similar expression levels while PBSC-NK cells had significantly higher DNAM-1 expression compared to frozen CBSC-NK cells ($p<0.05$, figure 4.18A).

Fresh CBSC-NK cells had low expression of integrin β7 compared to frozen CBSC-NK cells (although not statistically significant), whereas frozen CBSC and PBSC had similar expression (figure 4.18A).

L-selectin expression was similar among CBSC samples (1 ± 0.3% and 3.9 ± 2.3% for fresh and frozen CBSC-NK cells respectively) and PBSC-NK cells (17 ± 14.6%, p=0.1137 when compared to frozen CBSC-NK cells). LFA-1 expression was low among frozen CBSC-NK cells samples, PBSC-NK cells and fresh CBSC-NK cells showed a trend for higher expression (p=0.0649). When we compared the expression of these integrins between CBSC cultures using all cytokines or only IL-15 no significant differences were found (figure 4.18B).

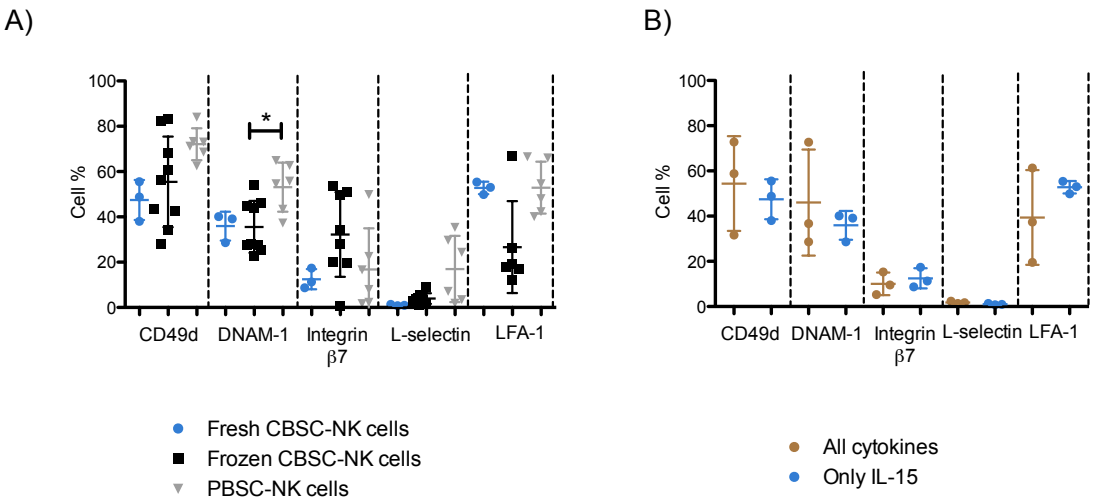


Figure 4.18. Expression of adhesion molecules receptors on NK cells. The graphs show the cell expression (mean ± SD) of CD49d, DNAM-1, Integrin β7, L-selectin and LFA-1 at day 35 on NK cells (percentages come from the $CD56^{+}CD3^{-}$ gate) from A) fresh CBSC (n=3), frozen CBSC (n=9), PBSC cultures (n=6) and B) CBSC cultures with all cytokines (n=3) or only IL-15 (n=3). Mann Whitney test was performed. * p<0.05.

Finally, the expression of adhesion molecules was analysed in the two different CD56 subsets in frozen CBSC and PBSC cultures. The expression of L-selectin and LFA-1 was found to be higher among $CD56^{dim}$ cells for frozen CBSC cultures (p<0.05, figure 4.19) compared to $CD56^{bright}$ cells.

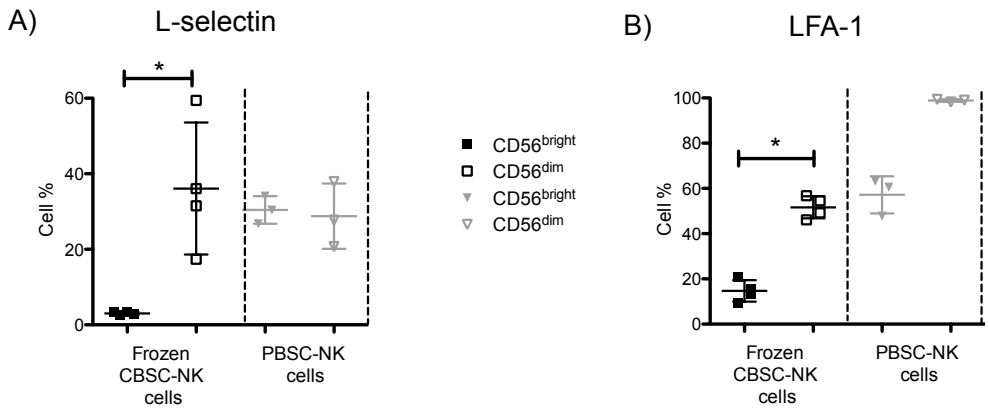


Figure 4.19. Expression of adhesion molecules receptors on CD56^{bright} and CD56^{dim} subsets. Graphs depict the expression at day 35 of L-selectin (A) and LFA-1 (B) on NK cells (percentages come from the CD56^{+}CD3^{-} gate) from frozen CBSC (n=4) and PBSC (n=3) cultures. Mann Whitney test was performed. * $p<0.05$.

In summary, NK cells generated *in vitro* expressed a wide variety of adhesion molecules with the exception of L-selectin. The expression of adhesion molecules will play an important role in NK cell-mediated killing, as these molecules are not only involved in adhesion but also in activating signalling.

4.3 Discussion

During this study high numbers of NK cells were generated. Although high numbers of NK cells are needed for NK cell immunotherapy, an optimal activation state is also critical. In this chapter a complete phenotypic characterisation was performed. Only a few studies performed a phenotypic characterisation of NK cells derived from CBSC (Spanholtz *et al.*, 2010; Lehmann *et al.*, 2012) or from PBSC (Yoon *et al.*; Zamai *et al.*, 2012). We believe the most complete characterisation of NK cells generated *in vitro* so far was achieved in this study. In addition, a complete characterisation of CB and PB NK cells was performed (appendix 1). The results of this publication were key in order to compare NK cell phenotype from different sources and are discussed in this chapter and also chapter 5.

While studying the final NK cell product, generated NK cells had some similarities with those of stage 3/4 described by Freud et al. (Freud *et al.*, 2006). Nevertheless, our approach integrates molecular data along with a detailed examination of marker expression in $CD56^{bright}$ and $CD56^{dim}$ cell subsets. The findings of this study suggest that the system used supports NK cell differentiation with a final product expressing activating receptors and low KIRs expression. Notably, the produced NK cells have a distinct phenotype that does not precisely resemble CB or PB NK cells.

The expression of KIRs and recently NKG2A has been linked to functional maturation and NK cell licensing/education (Kim *et al.*, 2005b; Anfossi *et al.*, 2006). The inhibitory receptors CD158a and CD158b are characteristics of NK cell stage 4/5. Even though stage 4 cells were present in all HSC cultures (chapter 3), a low expression of CD158a and CD158b in NK cells generated *in vitro* was observed. It is worth mentioning that the low expression observed (1-3%) was very similar to that described by other authors using either CBSC or PBSC (Yoon *et al.*; Bonanno *et al.*, 2009; Spanholtz *et al.*, 2010; Zamai *et al.*, 2012). Although no differences in KIRs expression between CBSC-NK and PBSC-NK cells were observed, it has been reported that there is a relationship between HSC source and KIR acquisition on NK cells. Miller *et al*. suggest that the capacity for obtaining KIR^{+} NK cells *in vitro* increased, as the ontogeny of HSC was more immature (foetal liver>CB>BM) (Miller and McCullar, 2001). Regardless of the low KIRs expression, the KIRs distribution on $CD56^{dim}$ and $CD56^{bright}$ subsets was analysed and a higher percentage in $CD56^{dim}$ cells was found. This percentage (10%) was not as high as in PB and CB $CD56^{dim}$ cells (20-50%) (Luevano *et al.*, 2012a). Nevertheless, this data correlates with current development models of NK cells indicating that $CD56^{dim}$ have a higher KIR expression (Andre *et al.*, 2000; Jacobs *et al.*,

2001). KIR2DL1 and KIR2DL2 mRNA expression was also investigated in PB NK cells, CB NK cells and NK cells generated in *vitro*. Surprisingly, KIR2DL1 and even KIR2DL2 mRNA expression in the generated NK cells was very similar to that of NK cells from CB and PB. Therefore, is there a missing signal or stimulus that will enable KIRs mRNA translation in NK cells generated *in vitro*? Dezell *et al*. used CBSC to generate NK cells *in vitro*; their results suggest that early signals from the feeder layer (from day 0-14) are needed for KIR acquisition (Dezell *et al.*, 2012). It could be the case that additional signals are required in our system for KIR acquisition, though all these signals have not yet been fully identified. In an effort to identify these signals, Cichocki *et al*. found that the transcription factor c-Myc enhances the transcription of KIR genes. Moreover, c-Myc binding was augmented upon IL-15 stimulation (Cichocki and Miller, 2010). Along these lines, IL-15 has been reported to induce KIR expression in mature NK cells *in vitro* (de Rham *et al.*, 2007) and *in vivo* (Huntington *et al.*, 2009). The cultures in this study included IL-15; for fresh CBSC cultures we maintained either a constant IL-15 concentration along with other factors (20 ng/ml) or only IL-15 after week 3 at a higher concentration (50 ng/ml). No differences in KIRs expression were observed between these cultures. Some studies have shown that IL-15 trans-presentation is needed at different stages for NK cell maturation, homeostasis and effector function acquisition (Huntington *et al.*, 2009; Lee *et al.*, 2011). We are not aware of any IL-15 trans-presentation in our system; perhaps this could be a reason why IL-15 did not increase KIRs expression in the generated NK cells.

Whether or not the absence of KIRs on NK cells generated *in vitro* could be an advantage for immunotherapeutic purposes still remains unknown in our model.
Recent studies have suggested that blocking KIR interactions with HLA-C ligands can enhance NK cell cytotoxicity; this can be achieved by the use of IPH2101 antibody (formerly 1-7F9), blocking KIRs2DL1, -2 and -3 in order to “uninhibit” NK cells and mount an anti-tumour response (Romagne *et al.*, 2009; Vey *et al.*, 2012). This “uninhibition” should be taken with care, as the risk of NK cell autoreactivity conveys a potential drawback for immunotherapy.

According to Grzywacz *et al*., the absence of expression of NKG2A and KIRs may suggest that NK cells are still immature (Grzywacz *et al.*, 2006). Moreover, Foley *et al*. suggested that the lack of inhibitory receptors on NK cells can result in hyporesponsiveness (Foley *et al.*, 2011b). However, the generated NK cells in this work expressed NKG2A, the only screened inhibitory receptor besides KIRs. NK cells expressed NKG2A at similar levels to those published by Spanholtz *et al*. (Spanholtz *et al.*, 2010; Spanholtz *et al.*, 2011a); other studies do not report NKG2A expression

(Yoon *et al.*; Zamai *et al.*, 2012). A further characterisation revealed that CD56^{dim} NK cells from frozen CBSC cultures had a higher NKG2A expression. Contrary to this data, our characterisation of CB and PB NK cell subsets showed a higher expression of NKG2A in CD56^{bright} NK cells (Luevano *et al.*, 2012a).

Studies using mice suggested that NKG2A could be responsible for the self-tolerance mechanism during NK cell development (Sivakumar *et al.*, 1999), whereas others claim that it is dispensable for NK cell education (Orr *et al.*, 2010). An increase in NKG2A mRNA expression throughout the culture was observed during this study, especially during the last three weeks. During NK cell development, NKG2A is expressed after NCRs and NKG2D. Indeed, at day 35, NCRs and NKG2D were expressed in the majority of the cells. CD56^{dim} cells had a higher NKG2A expression. From there, as NK cells mature, there should be a decrease of NKG2A expression and an increase of KIRs expression that is not occurring in our system (Bjorkstrom *et al.*). In line with these observations, when analysing NKG2A mRNA expression, a tendency for higher expression in NK cells generated *in vitro* compared to CB and PB NK cells was detected (Lutz *et al.*, 2011). Possibly a decrease in NKG2A expression along with KIRs acquisition will occur under different culture conditions or even *in vivo*.

According to the NK cell development model proposed by Freud *et al*., NK cells acquire NKp46 during stage 4; in this model NKp44 and NKp30 were absent (Freud *et al.*, 2006). NKp30 and NKp46 are expressed in resting and activated NK cells, whereas NKp44 is only expressed in activated NK cells (Pessino *et al.*, 1998; Vitale *et al.*, 1998; Pende *et al.*, 1999). In agreement with the results presented here, numerous reports show high expression of NKp30, NKp44 and NKp46 while generating NK cells *in vitro* (Yoon *et al.*; Perez *et al.*, 2006b; Spanholtz *et al.*, 2010; Beck, 2011; Spanholtz *et al.*, 2011a). Nevertheless, NKp80 (virtually expressed by all NK cells (Vitale *et al.*, 2001)) was absent in the generated NK cells; similar results were reported in another study (Spanholtz *et al.*, 2010). Due to the nature of the culture conditions, where the use of interleukins is standard (like IL-15), it is expected that NK cells generated *in vitro* will express NCRs (de Rham *et al.*, 2007). Likewise, a high NKp30 and NKp46 expression on CB and PB NK cells was observed. Contrary to CB and PB NK cells in which NKp46 expression is higher in CD56^{bright} cells (Luevano *et al.*, 2012a), a higher NKp46 expression in the CD56^{dim} subset from CBSC cultures was found. Along with this particular phenotype, in which there is low resemblance with either traditional CB or PB NK cells, we noticed the absence of expression of an important co-receptor; 2B4. 2B4 may exert inhibitory functions during the first NK cell development stages and progressively as the cell matures, plays a role as activating receptor (Sivori *et al.*,

2002). In addition, it has been proposed as a marker for NK cell lineage commitment (Eissens *et al.*, 2012). In general, other reports have shown the generation of NK cells with 2B4 expression in 20-30% (Perez *et al.*, 2006b) or 100% of NK cells (Beck *et al.*, 2009; Spanholtz *et al.*, 2010; Beck, 2011; Spanholtz *et al.*, 2011a). Additionally, the majority of PB and CB NK cells express 2B4 (Luevano *et al.*, 2012a). Although 2B4 was not detected by flow cytometry in NK cells generated *in vitro*, high mRNA expression at day 7 and 35 in CBSC cultures was observed. The levels in PBSC cultures remained constant and low during the whole culture. From here, we face two important questions: 1) Why were we unable to detect 2B4 by flow cytometry? 2) Does 2B4 play a different role in NK cell development from CBSC and PBSC? To answer the first question, it can be argued that commercial antibodies recognise specific epitopes. However, a characterisation in CB and PB NK cells using the same antibody was performed: the results showed optimal staining (Luevano *et al.*, 2012a). Antibodies recognise specific epitopes, either linear or conformational. The majority of the epitopes are conformational, where the recognised structure is formed by segments of proteins that are not continuous in the amino acid sequence, forming a 3-D structure. It could be that the conformational structure of 2B4 on NK cells generated *in vitro* is not recognised by the antibody used in this work. The clone PP35 was used, other reports use C1.7.1 or do not report the clone used. Certainly 2B4 mRNA was detected in all our cultures, CBSC samples had higher expression compared to PBSC. To answer the second question, our work cannot conclude whether 2B4 plays a different role in PBSC NK cells, as directed assays were not performed. Nevertheless, our results on CB and PB characterisation show that CB NK cells have a higher 2B4 expression compared to PB NK cells (Luevano *et al.*, 2012a). Interestingly, CD48 (2B4 ligand) was expressed in frozen CBSC-NK and PBSC-NK cells, while absent in fresh CBSC-NK cells. Interactions between 2B4-CD48 in NK cells have been reported to augment proliferation in response to IL-2 (Assarsson *et al.*, 2004). The reason for the absence of CD48 in fresh CBSC-NK cells remains unknown in our system. Regardless of the absence of 2B4 expression in all generated NK cells, it would be expected that CD48 might participate in adhesion and as co-stimulator for NK cell activation in frozen CBSC-NK and PBSC-NK cells.

Besides NCRs, there are also other receptors of major importance; the death receptor family (Fas-L and TRAIL) and CD94/NKG2 family (NKG2C and NKG2D). Fas-L and TRAIL are able to induce apoptosis of targets (Montel *et al.*, 1995; Takeda *et al.*, 2001). Differences in the expression of Fas-L between frozen CBSC-NK and PBSC-NK cells were found. The data obtained showed that unlike CB and PB NK cells (where Fas-L expression is around 10% (Luevano *et al.*, 2012a)), almost 100% of PBSC-NK

cells expressed Fas-L, whereas fresh and frozen CBSC-NK cells had lower expression (20-40%). Fas-L is usually expressed at very low and steady levels on NK cells; however, they express significant levels of intracellular Fas-L (Wallin *et al.*, 2003). In fact, the same report suggests that the ligation of MHC class-I prevents upregulation of cell surface Fas-L. The absence of KIRs expression and therefore the lack of interactions with MHC in our system could contribute to the abundant Fas-L expression observed. Similarly, TRAIL was expressed abundantly in all generated NK cells, especially frozen CBSC-NK and PBSC-NK cells. CB and PB NK cells express low levels of TRAIL, around 15% (Dalle *et al.*, 2005; Luevano *et al.*, 2012a). TRAIL can mediate apoptosis in tumour cells, contributes to NK cell-mediated killing of immature DCs (Hayakawa *et al.*, 2004) and is expressed in immature NK cells ($CD161^{+}CD56^{-}$) (Zamai *et al.*, 1998b). The high expression of this receptor on NK cells could be useful, a recent report shows that TRAIL overexpression on NK cells using zoledronic acid was able to augment cytotoxicity against TRAIL-sensitive tumour cells (Sarhan *et al.*, 2013). Overall, the high expression of Fas-L and TRAIL can be translated to a better killing capacity in a granule-independent manner.

Following the screening of activating receptors, the absence of NKG2C and expression of NKG2D on the generated NK cells was observed. Compared to PB NK cells, CB NK cells do not express NKG2C (Luevano *et al.*, 2012a). Likewise, other reports show the absence of expression of NKG2C on NK cells generated *in vitro* (Spanholtz *et al.*, 2010; Beck, 2011). Regardless of the surface expression, NKG2C mRNA levels were very similar between NK cells generated *in vitro* and CB and PB NK cells. Lehemann *et al*. found low levels of activating NKG2C mRNA on NK cells generated *in vitro* (Lehmann *et al.*, 2012). It seems that NKG2C expression is very variable among samples; it has been associated with HCMV seropositivity (Guma *et al.*, 2006a). In that sense, the lack of NKG2C expression would be expected due to the nature of our culture, where NK cells were not exposed to HCMV.

Another important activating receptor and perhaps one of the best characterised is NKG2D. Almost 100% of CB and PB NK cells express NKG2D (Freud *et al.*, 2006; Luevano *et al.*, 2012a). In agreement with our observations, the majority of the NK cells generated *in vitro* expressed NKG2D (Yoon *et al.*; Spanholtz *et al.*, 2010; Beck, 2011; Spanholtz *et al.*, 2011a). This receptor plays a key role in the control of infected and transformed cells and recently it was suggested to play an important role in NK cell development (Zafirova *et al.*, 2011). The expression of NKG2D on the generated NK cells suggests the possibility of tumour recognition and potential killing of jeopardised cells infected by virus or DNA damage.

Bryceson and colleagues studied the combination of activating receptors needed to elicit cytotoxicity or cytokine secretion; their work concluded that none of these receptors could trigger a response alone, except CD16 (Bryceson *et al.*, 2006b). Differences in the level of expression of CD16 have an impact on ADCC functions. ADCC is an effective mechanism in which CD16 on NK cells recognises the Fc portion of IgG antibodies bound to malignant cells. Almost 100% of PB NK cells and around 50% of CB NK cells (highly variable) express CD16 (Luevano *et al.*, 2012a). The majority of protocols that generate NK cells *in vitro* give rise to $CD56^{bright}CD16^{-/low}$. During this work, a small fraction (10-25%) of NK cells expressing CD16 was detected. Few studies report the generation of $CD56^{+}CD16^{+}$ cells, either using PBSC (100% $CD16^{+}$) (Giuliani *et al.*, 2008) or CBSC (24% $CD16^{+}$) (Frias *et al.*, 2008). CD16 acquisition depends on the type of system and conditions used. Dezell *et al*. found a higher CD16 percentage in NK cells generated using a heparin-based system compared to the use of the feeder layer EL08.1D2 (Dezell *et al.*, 2012). The absence of CD16 in the majority of the generated NK cells predicts a deficient ADCC activity. Nevertheless, we believe that additional signals would be provided *in vivo* that will support NK cell maturation and therefore CD16 acquisition.

While studying the phenotype of the generated NK cells *in vitro* we observed the expression of abundant activating receptors and low expression of KIRs. In addition, the characterisation of interleukin receptors on NK cells generated *in vitro* was included in this study. Four out of the five investigated interleukin receptors were expressed differentially between fresh and frozen CBSC-NK cells. Conversely, only IL-18R was differentially expressed between frozen CBSC-NK cells and PBSC-NK cells. Frozen CBSC-NK cells expressed more IL-2Rα and IL-2Rβ1 and less IL-15Rα and IL-12Rβ1 (IL-12Rβ1 had increased expression in the $CD56^{bright}$ subset) compared to their fresh counterpart. Like CB and PB $CD56^{bright}$ cells (Luevano *et al.*, 2012a), frozen CBSC-NK cells and PBCS-NK cells should have capacity to respond to IL-2 due to CD25 and CD122 expression (IL-2Rα and IL-2Rβ1, respectively). This profile resembles that of $CD56^{bright}$ cells, providing a better proliferation and activation capacity, as IL-2 effects on NK cells have been well studied (Poli *et al.*, 2009). On the other hand, the absence in fresh CBSC-NK cells of the high affinity IL-2 receptor (CD25) might indicate a more mature stage, resembling the $CD56^{dim}$ subset. Fresh CBSC-NK cells might have a better response to IL-12 and IL-15, therefore producing more IFN-γ, increasing survival and enhancing NK cell cytotoxicity (Kobayashi *et al.*, 1989; Naume *et al.*, 1992; Gamero *et al.*, 1995; Carson *et al.*, 1997). The overall repertoire of interleukin receptor expression direct us toward the assumption that fresh CBSC-NK cells resemble the

$CD56^{dim}$ subset while frozen CBSC-NK cells and PBSC-NK cells resemble the $CD56^{bright}$ subset. Finally, the expression of IL-18R is more common in the $CD56^{bright}$ subset (Poli *et al.*, 2009); interestingly it was expressed in PBSC-NK cells (increased expression in the $CD56^{dim}$ subset) and not CBSC-NK cells (fresh and frozen). IL-18 is produced by macrophages during infection, which synergises with other factors in order to increase IFN-γ production (Sareneva *et al.*, 1998; Strengell *et al.*, 2003). The lack of IL-18R on CBSC-NK cells could thus impact on IFN-γ production; nevertheless, other cytokines like IL-12 induce its expression (Sareneva *et al.*, 2000).

Until recently, very little was known about NK cell migration and trafficking. Current research has implicated that chemokine receptors are able to control migration and functions of NK cells. Because the different CD56 subsets have unique distribution and function, their chemokine receptor profile is also different. $CD56^{bright}$ cells express little CXCR1, CXCR2 and CXCR3 but high levels of CCR5 and CCR7 (Robertson, 2002). $CD56^{dim}$ cells express CXCR1, CXCR2, CXCR3 and CXCR4 but not detectable levels of CC receptors (Robertson, 2002). Our characterisation showed a higher expression of CXCR1 and lower CXCR4 in PB $CD56^{dim}$ cells (Luevano *et al.*, 2012a). CB $CD56^{dim}$ cells also showed a higher expression of CXCR1, but CXCR4 had a higher expression on $CD56^{dim}$ cells (Luevano *et al.*, 2012a). The chemokine receptor characterisation will vary according to staining protocols, clones used and taking into consideration the status of NK cells, either resting or activated. We allowed 2-3 h resting of the cells before all cell-surface labelling. Therefore, a direct comparison with other studies was difficult. During this work the expression of the majority of the chemokine receptors was very variable, especially in the frozen cultures (CBSC and PBSC). Fresh CBSC-NK cells had very low or no chemokine receptor expression at all. Because of the high variability, only the chemokine receptors that had no variation among samples will be discussed; namely CCR7, CXCR7 and CXCR4. The absence of CCR7, frequently expressed in $CD56^{bright}$ cells (Poli *et al.*, 2009), suggests these cells will not be able to migrate to the lymph nodes (Campbell *et al.*, 2001). LNs are the site of interaction between different types of cells. DCs upregulate CCR7 after encountering a pathogen, then, migration to lymph nodes occurs, followed by interaction with $CD56^{bright}$ cells and T cells (Palucka and Banchereau, 2002). NK cells can induce maturation of DCs, either by secretion of IFN-γ and TNF or in a cell-cell dependent manner (Gerosa *et al.*, 2002). However, this cross-talk between NK cells and DCs can also lead to blocking of antigen presentation (Gerosa *et al.*, 2002). The lack of CCR7 could be taken as a sign of NK cell maturity.

In the case of CXCR7, inconsistent results have been reported (Hartmann *et al.*, 2008; Berahovich *et al.*, 2010a). In agreement with our data, a recent study suggests that NK cells do not express CXCR7 (Berahovich *et al.*, 2010a; Berahovich *et al.*, 2010b). Fresh CBSC-NK cells did not express CXCR4, but frozen CBSC-NK cells and PBSC-NK cells did. Nevertheless, this observation deserves further investigation, as this work did not include migration assays. Furthermore, we found that $CD56^{bright}$ cells from frozen CBSC cultures expressed more CXCR4. Some researchers propose that CXCR4 is expressed by both PB CD56 subsets (Robertson, 2002), others have found CXCR4 to be more expressed in PB $CD56^{dim}$ cells (Campbell *et al.*, 2001) or CB $CD56^{dim}$ cells (Luevano *et al.*, 2012a). As previously mentioned, there are many discrepancies in the literature due mainly to different cell isolation procedures, staining protocols and even the antibodies used.

In addition to signals for tissue-specific migration, NK cells need to undergo additional steps in order to carry out their functions: migration and cell adhesion. NK cells are equipped with a variety of integrins and adhesion molecules. For instance, CD49d (integrin α4) mediates cell to cell and cell to extracellular-matrix interactions, and is also known to be crucial for transendothelial migration (Humphries *et al.*, 1995). NK cells from fresh CBSC and CBSC and PBSC frozen cultures expressed CD49d to a lesser extent compared to PB and CB NK cells (Luevano *et al.*, 2012a). The expression of integrin β7 was less compared to CD49d, especially in fresh CBSC-NK cells. Regardless, integrin β7 expression in all NK cells generated *in vitro* was higher compared to that of CB and PB NK cells (Luevano *et al.*, 2012a). Integrin β7 forms heterodimers with CD49d (integrin α4) and plays an important role in interaction with both extracellular matrix and endothelial cells (Perez-Villar *et al.*, 1996). According to our characterisation, integrin β7 will be able to form heterodimers thanks to the availability of CD49d. We did not screen for integrin β1, another integrin that can bind to CD49d. However, CD49d is potentially shared by these two integrins. Our results suggest that the generated NK cells may be able to interact with the extracellular matrix and with other cells.

Another cell adhesion molecule studied was L-selectin, which facilitates the migration of lymphocytes from blood into secondary lymphoid tissues (Bevilacqua *et al.*, 1991). Similar to our results, it has been reported that PB $CD56^{bright}$ cells express L-selectin (Frey *et al.*, 1998; Luevano *et al.*, 2012a), whereas CB NK cells ($CD56^{bright}$ or $CD56^{dim}$ subsets) do not (Luevano *et al.*, 2012a). As NK cells mature they lose L-selectin expression (Andre *et al.*, 2000). During this work, the absence of this molecule in CBSC cultures and very low expression in PBSC-NK cells was detected. This profile

resembles that of CB NK cells (Luevano *et al.*, 2012a). The absence of L-selectin reaffirms the probable inability of the generated NK cells to migrate to lymphoid tissues. The questions to address would be: 1) can NK cells shape their repertoire and acquire molecules that allow homing to LNs? 2) What would be the consequences if the generated NK cells cannot home to LNs? There are some studies reporting that NK cell phenotype is shaped by the microenvironment (Di Santo, 2008; Shi *et al.*, 2011). Regarding the consequences, studies in HIV infected individuals have shown the presence of $CCR7^{-}CD56^{bright}$ cells; moreover, these cells can be present in healthy individuals but in low frequencies (Hong *et al.*, 2012). These cells have a high cytotoxic activity, with mature phenotype and an activated state. Keeping in mind that our work does not include migration assays, we suggest that the expression of L-selectin and CCR7 for homing to lymph nodes will be shaped according to the physiological needs.

Adhesion molecules are important not only for cellular binding, but also for signal transduction (Hogg and Landis, 1993). For instance, LFA-1 is required for efficient lysis of target cells through cell adhesion in addition to initiating activating signals in NK cells (Barber *et al.*, 2004; Mace *et al.*, 2010). NK cells from fresh CBSC and PBSC had a tendency for higher expression of LFA-1, suggesting that lysis could be facilitated at a greater extent compared to NK cells from frozen CBSC cultures. It has been shown that LFA-1 interacts with DNAM-1 (also known as CD266) and was expressed by all PB NK cells and to a lesser extent by CB NK cells (Luevano *et al.*, 2012a). DNAM-1 is an adhesion molecule that can trigger NK cell cytotoxicity. Shibuya *et al*. reported that DNAM-1 activation is dependent upon functional interaction with LFA-1 (Shibuya *et al.*, 1999). We observed a higher expression of DNAM-1 in PBSC-NK cells compared to CBSC-NK cells. The levels of LFA-1 in CBSC $CD56^{dim}$ cells were higher compared to the $CD56^{bright}$ subset. Altogether these data suggest that PBSC-NK cells have higher levels of LFA-1 and DNAM-1, indicating a probable advantage over CBSC-NK cells (fresh and frozen).

Figure 4.20 depicts the complete phenotypic characterisation of NK cells generated *in vitro* from fresh and frozen CBSC cultures. Little differences were found when using fresh or frozen CBSC for the majority of the NK cell receptors tested. Overall, the most interesting differences were the tendencies for a lower expression of CD16 and LFA-1 and a higher expression of TRAIL, CXCR4 and integrin β7 in frozen CBSC-NK cells compared to fresh CBSC-NK cells. The mechanisms responsible for these differences are unclear. The low expression of LFA-1 and CD16 and higher expression of CXCR4 and integrin β7 in frozen CBSC-NK cells suggests an immature phenotype (Freud *et al.*, 2006; Zamai *et al.*, 2009; Montaldo *et al.*, 2012).

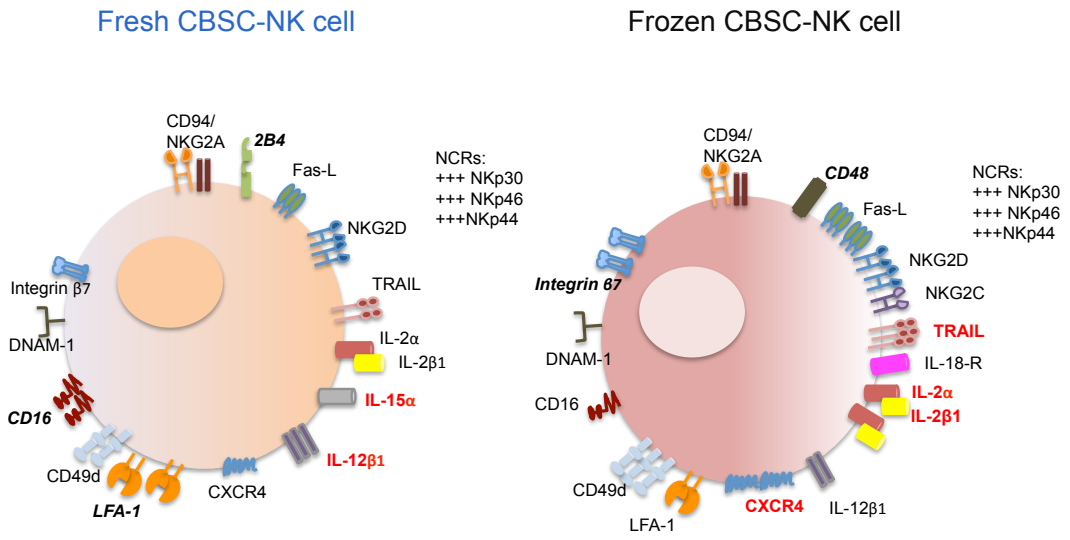


Figure 4.20. Phenotypic characterisation of CBSC-NK cells. The figure shows the characterisation of fresh CBSC-NK cells and frozen CBSC-NK cells. The markers in bold red are significantly higher compared to their counterpart. The markers in italics and bold black are different but not statistically significant.

Conversely, fewer differences in the phenotype between frozen CBSC-NK and PBSC-NK cells were found. CBSC-NK cells expressed more TRAIL and less DNAM-1, Fas-L and IL-18-R (Figure 4.21). This suggests that PBSC-NK cells could have a better killing capacity due to the expression of adhesion/activation molecule DNAM-1 and death receptor Fas-L.

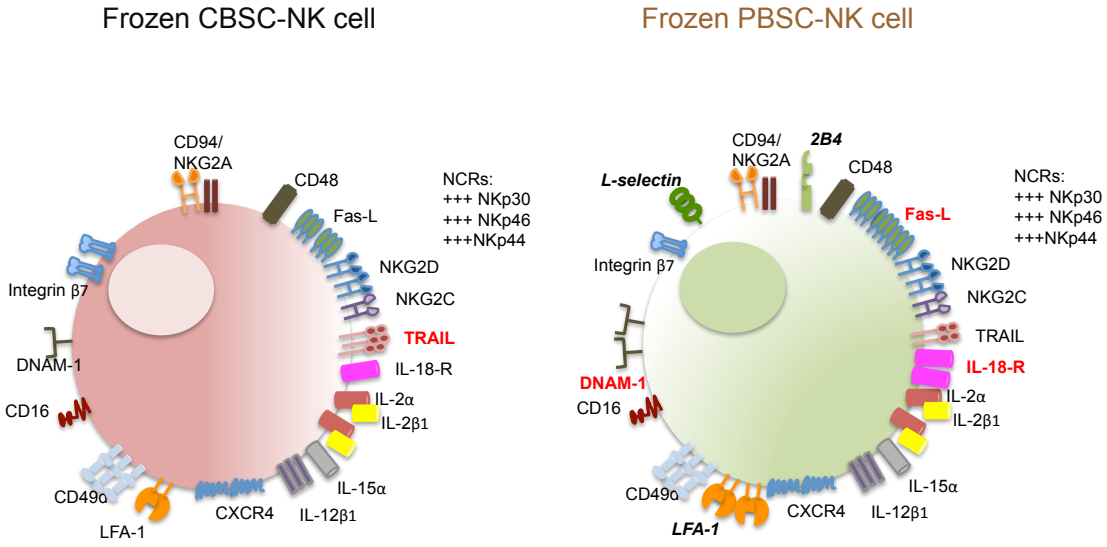


Figure 4.21. Phenotypic characterisation of frozen CBSC and PBSC NK cells. The figure depicts the phenotype of frozen CBSC-NK cells and PBSC-NK cells. The markers in bold red are significantly higher compared to their counterpart. The markers in italics and bold black are different but not statistically significant.

Although NK cells generated *in vitro* do not precisely resemble either PB or CB NK cells, the expression of some receptors like CXCR4 and integrin β7 or lack of KIRs suggest that the generated NK cells could behave like $CD56^{bright}$ cells. The question would now be: would a $CD56^{bright}$ cell phenotype be a disadvantage? Just recently, a study followed NK cell turnover in adults; the results suggested that $CD56^{bright}$ NK cells were able to proliferate rapidly and die slowly compared to $CD56^{dim}$ (Lutz *et al.*, 2011). In addition, Lehmann *et al*. suggested that *in vitro* generated NK cells that resemble $CD56^{bright}$ NK cells display a gene expression profile similar to those of $CD56^{dim}$ NK cells in PB (Lehmann *et al.*, 2012). This data is in accordance to our study where we found that mRNA levels for most of the markers are expressed similarly or even at higher levels compared to CB and PB NK cells. Altogether, these data support the notion that $CD56^{bright}$ NK cells could be a good option for immunotherapeutic uses. These cells will have several advantages over $CD56^{dim}$ cells: higher proliferation and survival rates and the probability of better killing due to less inhibitory receptors expression.

The dynamic process of acquisition of NK cell receptors makes it difficult to assess if the phenotype of the generated NK cells will remain after infusion. The most probable outcome will be a change of this repertoire according to the environment. Nevertheless, it is important to carry out this characterisation, as it will provide information that will allow a correlation with the outcome of the NK cell therapy.

To summarize, we achieved one the most complete characterisation of NK cells generated *in vitro*. Furthermore, NK cells generated *in vitro* express NCRs, NKG2D and DNAM-1, cell markers that contribute to tumour killing according to Alici *et al*. (Alici *et al.*, 2008).

The findings discussed in this chapter were:

1) Frozen CBSC-NK cells had a similar phenotype compared to fresh CBSC-NK cells and even to PBSC-NK cells.
2) Evidence that removal of IL-15 for the last 3 weeks did not affect the phenotype of the generated NK cells was provided.
3) NK cells were generated that expressed activating receptors and low KIRs expression, but also expressed NKG2A and were potentially capable of BM homing and responding to different cytokines.

4) This phenotype does not precisely resemble CB or PB NK cells; instead, NK cells generated *in vitro* have a unique phenotype and a similar transcriptional profile.

Chapter 5: Functionality of Natural Killer cells generated *in vitro*

5.1 Introduction

NK cells are able to perform their function through two important mechanisms: production of cytokines such as IFN-γ, TNF-α, GM-CSF and cytotoxicity mediated via granule-secretion of perforin/granzyme or via death-receptors. NK cell cytotoxic and immunoregulatory functions have been widely studied, both in CB and PB. It is well established that NK cell subsets have different phenotype and functions (Cooper *et al.*, 2001a). Studies have revealed the different cytotoxic potential held by $CD56^{bright}$ and $CD56^{dim}$ NK cells in CB and PB. $CD56^{bright}$ NK cells from both sources exhibit low cytotoxicity (Cooper *et al.*, 2001b) and CB $CD56^{dim}$ NK cells are considerably less cytotoxic than their PB counterpart (Tanaka *et al.*, 2003; Luevano *et al.*, 2012a). Moreover, resting CB NK cells are more naïve and heterogeneous compared to PB NK cells (Hoshina *et al.*, 1999; Tanaka *et al.*, 2003). CB NK cells have been shown to have a more immature phenotype (Luevano *et al.*, 2012a), and to exhibit a higher expression of inhibitory receptors (Wang *et al.*, 2007) and lower expression of adhesion molecules (Tanaka *et al.*, 2003), features that could contribute to the lower CB NK cell cytotoxicity observed in different studies. Nevertheless, activation with IL-2, IL-12 or IL-15 can restore CB NK cell functions (Hoshina *et al.*, 1999; Luevano *et al.*, 2012a). These observations raise important questions: if CB NK cells are naïve and immature, would NK cells generated *in vitro* from CBSC have similar characteristics? Would NK cells generated from different HSC sources have different phenotype and effector functions as observed for PB and CB NK cells? The answers to these questions may influence decision as to which HSC source should be used for NK cell generation *in vitro*.

Efforts have been made to characterise effector functions of NK cells generated *in vitro*. In the majority of reports using CBSC (Kao *et al.*, 2007; Spanholtz *et al.*, 2010; Beck, 2011; Spanholtz *et al.*, 2011a; Dezell *et al.*, 2012) or PBSC (Woll *et al.*, 2005; Giuliani *et al.*, 2008; Woll *et al.*, 2009) NK cell effector functions were studied using a standard ^{51}Cr release assay and K562 cells. Some reports included the assessment of intracellular IFN-γ, granzyme B and perforin (Woll *et al.*, 2009; Lehmann *et al.*, 2012; Zamai *et al.*, 2012), very few included cytokine secretion or degranulation assays (Bonanno *et al.*, 2009; Spanholtz *et al.*, 2010; Spanholtz *et al.*, 2011a) and only one performed ADCC assays (Lehmann *et al.*, 2012) (see appendix 3). Furthermore, the

literature regarding the functional study of NK cells generated *in vitro* comparing different HSC sources is scarce (Carayol *et al.*, 1998; Woll *et al.*, 2009). For instance, Woll et al. compared the use of hESC to CBSC for NK cell generation *in vitro* (Woll *et al.*, 2009) and reported that CBSC-NK cells displayed low cytotoxicity and an immature phenotype compared to NK cells from hESC (Woll *et al.*, 2009). If indeed there is a difference in effector functions of NK cells generated *in vitro* according to HSC source, choosing the right HSC source will therefore be crucial as this will impact on the clinical outcome. A complete study including not only phenotypic but also functional characterisation of NK cells generated *in vitro* from different HSC source will help address this question.

Each protocol for NK cell generation *in vitro* has been optimised with cytokine cocktails that will vary in composition. Components in the media and the use or omission of a feeder layer for HSC culture will determine the acquisition of receptors on the generated NK cells and thus their function. Although many comparisons are probably performed while optimising a protocol, very few have been published (McCullar *et al.*, 2008; Dezell *et al.*, 2012). During this work, a functional analysis of NK cells generated *in vitro* using a modified published protocol was included (Grzywacz *et al.*, 2006).

In this chapter we performed what we believe is one of the most complete characterisations in terms of functionality of NK cells generated *in vitro*. We tested the impact of using a modified protocol on the generated NK cells *in vitro*, in addition to determining whether the use of cryopreserved CBSC cells would jeopardise NK cell functions. Finally, this complete characterisation was also performed using PBSC and compared to CBSC. This chapter includes a variety of assays to determine the cytotoxic and immunoregulatory properties of NK cells generated *in vitro*. The results of this chapter complement the phenotypic characterisation and provide a more thorough characterisation of NK cells generated *in vitro*.

5.2 Results

5.2.1 Frozen CBSC-NK cells secrete high amounts of IFN-γ and TNF-α

5.2.1.1 IFN-γ

In order to assess the functional features of NK cells generated *in vitro* a variety of assays were performed. NK cell subsets have different immunoregulatory and cytotoxic properties; $CD56^{bright}$ cells appear to have an intrinsic capacity to produce high levels of cytokines compared to $CD56^{dim}$ cells. Thus, immunoregulatory properties have been attributed to $CD56^{bright}$ NK cells (Cooper *et al.*, 2001a). IFN-γ is one of the main cytokines secreted by NK cells and can lead to activation of other cells (Cooper *et al.*, 2001b). In fact, IFN-γ is the primary macrophage-activating factor leading to increased tumour cytotoxicity, anti-microbial activity as well as antigen processing and presentation to lymphocytes (Young and Hardy, 1995). In addition, IFN-γ acts on $CD4^+$ T cell development, affecting the balance between Th1 and Th2 sub-populations (Young and Hardy, 1995). Although $CD56^{bright}$ cells have been shown to secrete high levels of IFN-γ a recent report suggests that $CD56^{dim}$ cells can also (De Maria *et al.*, 2011).

Due to the important role of IFN-γ, the secretion of this cytokine by NK cells generated *in vitro* was analysed. Supernatants of non-stimulated NK cells or NK cells stimulated for 2 h with K562 or Raji (only for fresh samples) and PMA&Iono (for all samples) were collected and used for ELISA analysis (figure 5.1). A minimal secretion of IFN-γ when cells were stimulated with K562 or RAJI cells was observed (range from 17.5 - 180 pg/ml). However, figure 5.2A shows that secretion of IFN-γ using PMA&Iono was significantly higher in NK cells from frozen CBSC cultures compared to fresh CBSC and to PBSC cultures (4180.4 ± 1848 pg/ml for frozen CBSC, 188.7 ± 101.7 pg/ml and 180.3 ± 244 pg/ml for fresh CBSC and PBSC respectively, $p<0.05$). No differences were observed between NK cells from fresh CBSC cultures using all cytokines or only IL-15 (figure 5.1B).

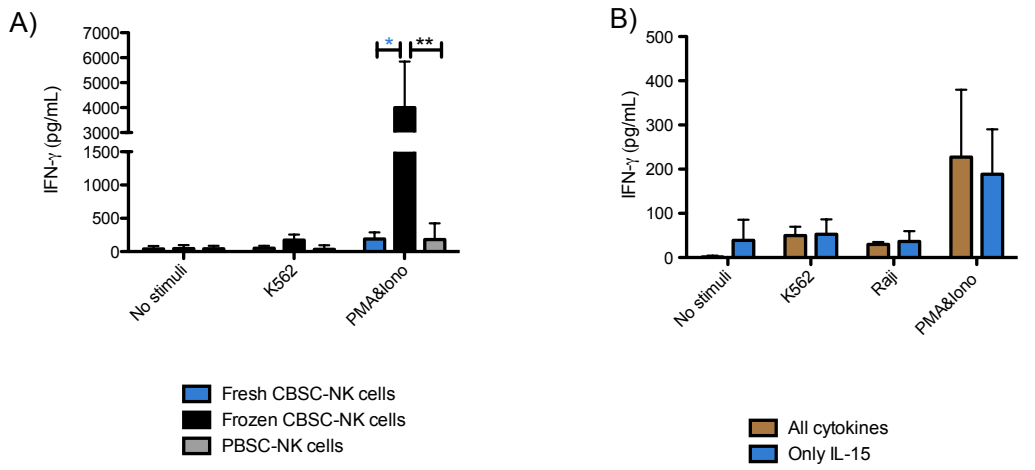


Figure 5.1. IFN-γ secretion of NK cells generated *in vitro*. A) Secreted IFN-γ by NK cells as measured by ELISA without stimuli, or stimulated with the cell lines K562 or Raji or with PMA&Iono for fresh CBSC-NK cells (n=3), frozen CBSC-NK cells (n=7), PBSC-NK cells (n=6) and NK cells from CBSC cultures with all cytokines (n=3) or only IL-15 (n=3). Mann Whitney test was performed. * $p<0.05$, ** $p<0.01$.

Regardless of the striking difference in secreted IFN-γ by frozen CBSC-NK cells, intracellular IFN-γ expression did not significantly differ between frozen CBSC-NK cells (23.1 ± 7.5%) and fresh CBSC-NK cells (35.6 ± 8.6%), or even between frozen CBSC-NK and PBSC-NK cells (17.7 ± 2.9%) (figure 5.2A). Similarly, there were no differences in intracellular IFN-γ expression between fresh CBSC-NK cells (35.6 ± 8.6% versus 31.8 ± 0.9%, all cytokines and only IL-15, respectively, figure 5.2B).

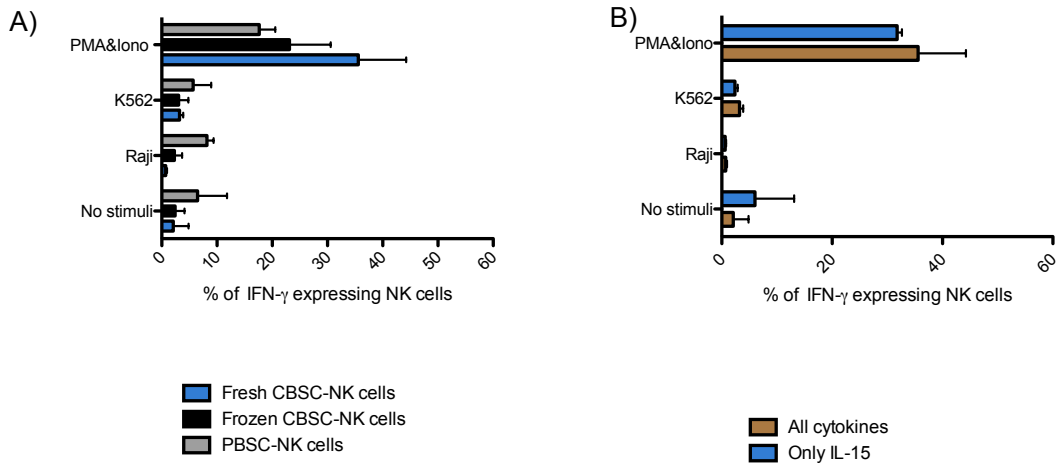


Figure 5.2. Intracellular IFN-γ expression in NK cells generated *in vitro*. Intracellular expression of IFN-γ analysed by flow cytometry in NK cells stimulated with PMA&Iono, K562 or Raji cells in the presence of GolgiStop™. A) Expression of IFN-γ for NK cells (percentages come from the $CD56^{+}CD3^{-}$ gate) from fresh (n=3) and frozen (n=9) cultures, PBSC-NK cells (n=6) and B) NK cells from CBSC cultures with all cytokines (n=3) or only Il-15 (n=3).

Flow cytometry data for intracellular IFN-γ revealed that there was no specific NK cell subset producing IFN-γ according to the CD56 surface density expression (figure 5.3 and 5.4).

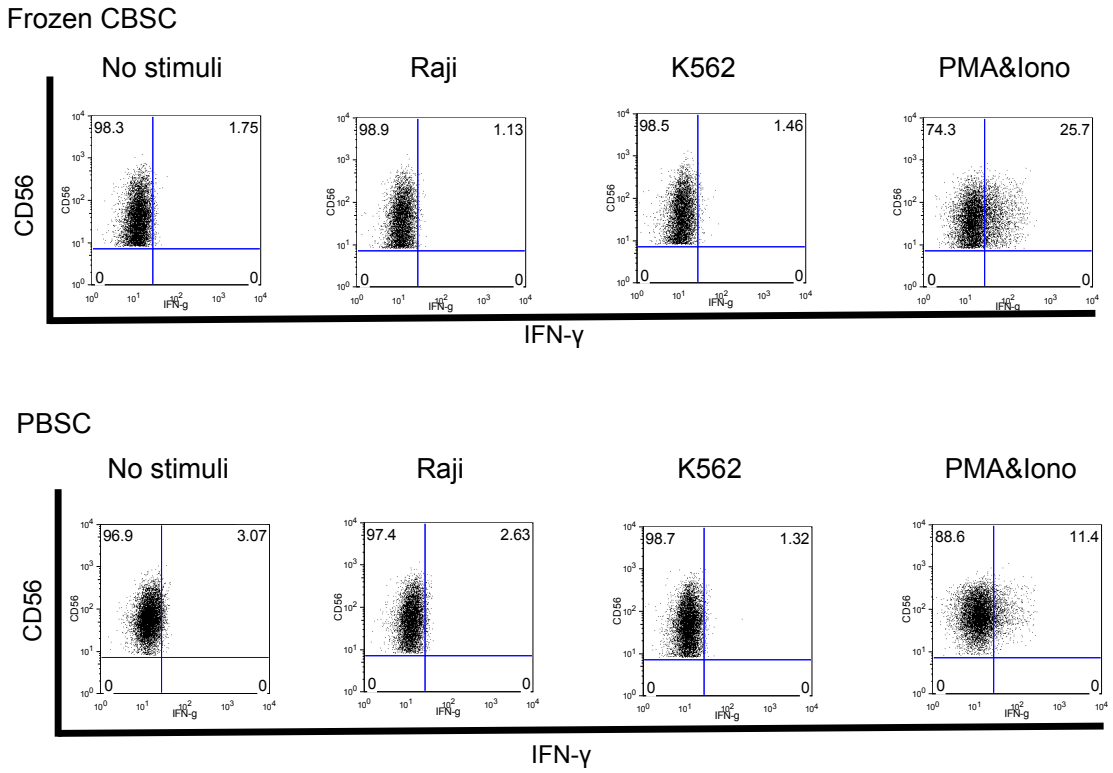


Figure 5.3. Flow cytometry analysis of intracellular IFN-γ in NK cells generated *in vitro*. Flow cytometry analysis showing a representative sample of frozen CBSC-NK cells (upper panel) and PBSC-NK cells (lower panel). The plots show IFN-γ versus CD56 expression from the lymphocyte gate in NK cells without stimuli or in the presence of cell lines Raji, K562 or PMA&Iono.

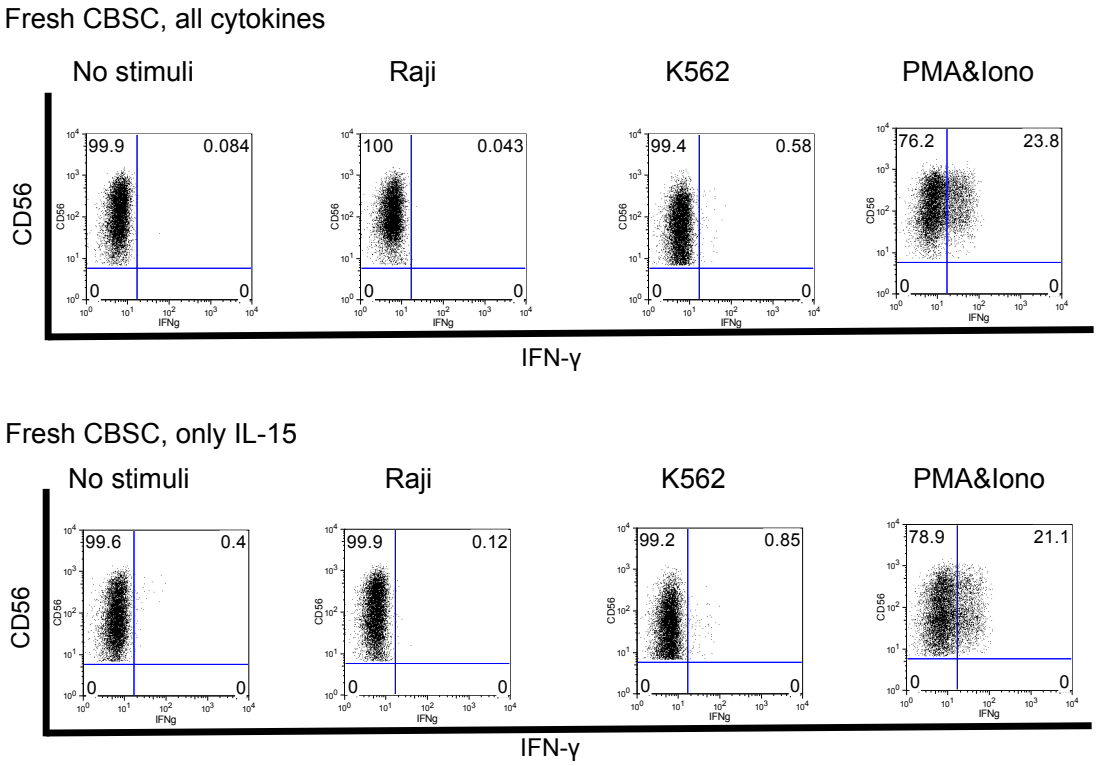


Figure 5.4. Flow cytometry analysis of intracellular IFN-γ in NK cells generated *in vitro*. Flow cytometry analysis showing a representative sample of fresh CBSC-NK cells using all cytokines (upper panel) or only IL-15 (lower panel). The plots show IFN-γ versus CD56 expression from the lymphocyte gate in NK cells without stimuli or in the presence of cell lines Raji, K562 or PMA&Iono.

The intracellular data revealed no differences in intracellular IFN-γ expression, however IFN-γ secretion was considerably higher in frozen CBSC-NK cells. Thus, IFN-γ mRNA levels were studied in NK cells from fresh CBSC and frozen CBSC. Additionally, IFN-γ mRNA expression in resting NK cells from CB and PB were included. Figure 5.5 shows the relative expression of IFN-γ mRNA in resting NK cells from CB, PB, fresh CBSC and frozen CBSC cultures. Although no significant differences were found between cultures, there was a tendency for a higher expression of IFN-γ mRNA in frozen CBSC-NK cells compared to PB NK cells (figure 5.5, p= 0.057).

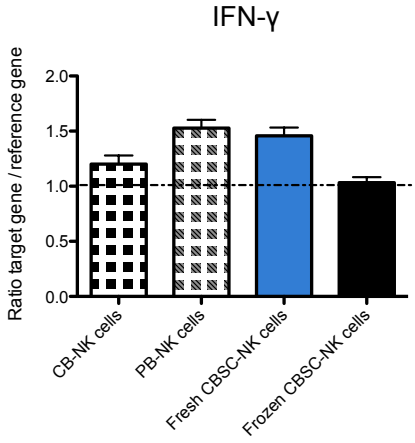


Figure 5.5. **mRNA analysis of IFN-γ.** Total RNA was extracted from resting NK cells from CB (n=3) and PB NK cells (n=3), and fresh CBSC (n=3) and frozen CBSC-NK cells (n=4) harvested at day 35. The graph depicts the ratio of IFN-γ and the reference genes expression.

Altogether, frozen CBSC-NK cells demonstrated to have a remarkable capacity to secrete IFN-γ. Regardless of the similar intracellular IFN-γ expression, CBSC-NK cells are able to secrete vast amounts of this cytokine as confirmed by ELISA and the detection of IFN-γ mRNA in the generated NK cells.

5.2.1.2 TNF-α

Another important cytokine secreted by NK cells is TNF-α. TNF-α is a cytokine able to regulate other immune cells, such as T cells (Scheurich *et al.*, 1987). Similar to IFN-γ, low TNF-α secretion was found when NK cells from all HSC cultures were stimulated with K562 cells; the secretion was even lower with Raji cells (figure 5.6A and B). NK cells stimulated with PMA&Iono from frozen CBSC cultures had significantly higher TNF-α expression compared to fresh CBSC cultures ($p<0.05$, figure 5.6A). NK cells from PBSC cultures had a variable secretion of TNF-α and no differences were found when compared to frozen CBSC-NK cells (figure 5.6A). Similarly, no significant differences between NK cells from fresh CBSC-NK cultures using all cytokines or only IL-15 were found (figure 5.6B).

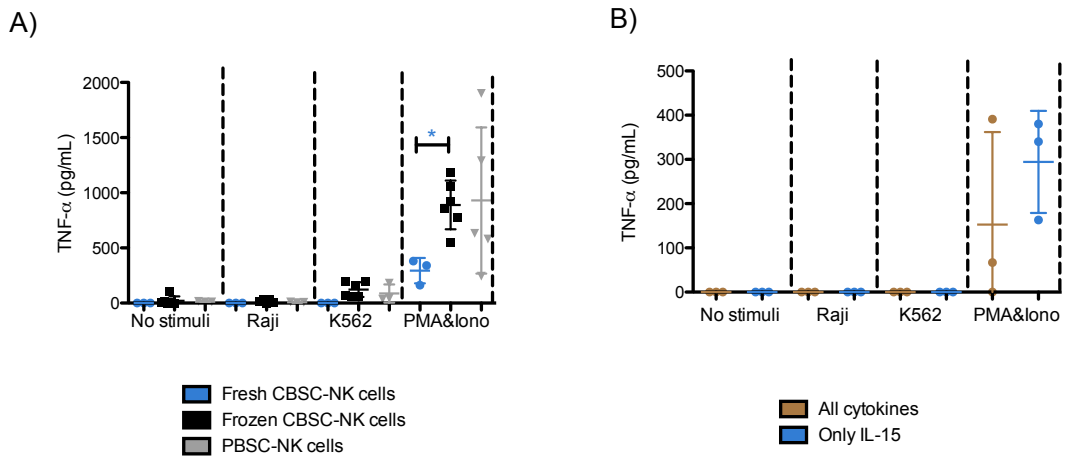


Figure 5.6. TNF-α secretion in the generated NK cells. A) Secreted TNF-α by NK cells as measured by ELISA without stimuli, or stimulated with the cell lines K562 or Raji and with PMA&Iono for fresh CBSC-NK cells (n=3), frozen CBSC-NK cells (n=6), PBSC-NK cells (n=3) and NK cells from CBSC cultures with all cytokines (n=3) or only IL-15 (n=3). Mann Whitney test was performed. * $p<0.05$,.

In summary, CBSC-NK cells showed higher TNF-α secretion compared to fresh CBSC-NK cells in response to PMA&Iono, whereas TNF-α secretion was similar between CBSC-NK cells and PBSC-NK cells.

5.2.2 Natural Killer cells from haematopoietic stem cell cultures degranulate against K562 cells and after stimulation with PMA&Iono

NK cells are able to kill via exocytosis of their granules or via death-receptor binding. CD107a is a membrane protein (LAMP-1) associated with lysosomes residing in cytolytic granule membranes located within the cytoplasm. CD107a is mobilised to the cell surface following activation-induced granule exocytosis. A degranulation assay detecting CD107a when NK cells generated *in vitro* were exposed to cell lines K562 and Raji, or stimulated with PMA&Iono was performed during this study. A high percentage of NK cells from all HSC cultures degranulated when stimulated with PMA&Iono (range 41-56%) and K562 cells (11.9-23.7%) but not Raji cells (around 3%, figure 5.7A and B). Although no significant differences between cultures were observed, the flow cytometry analysis showed that a stronger response was observed in fresh CBSC-NK cells (figure 5.8, PMA&Iono).

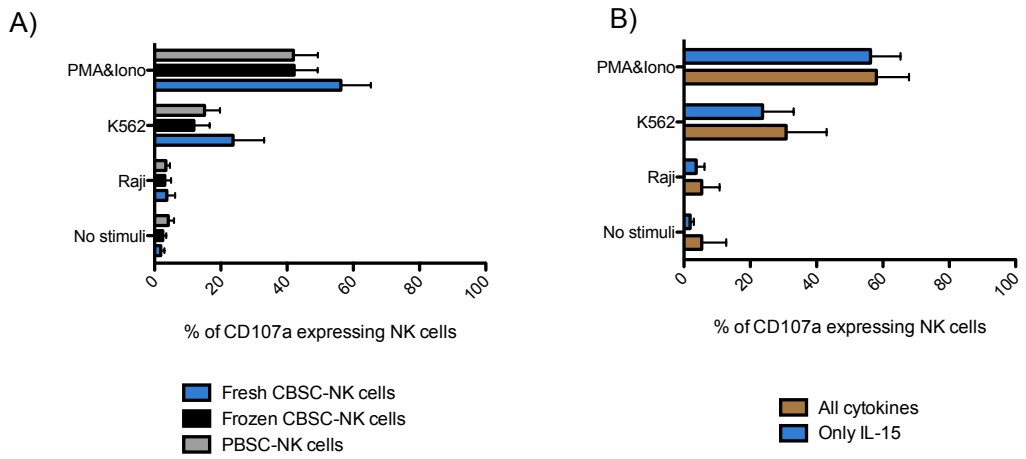


Figure 5.7. CD107a degranulation assay. Degranulation assay using $CD56^{+}CD3^{-}$ cells from the lymphocyte gate stimulated with PMA&Iono, K562 or Raji cells from A) fresh CBSC-NK cells (n=3), frozen CBSC-NK cells (n=6), PBSC-NK cells (n=6) and B) NK cells from CBSC cultures with all cytokines (n=3) or only IL-15 (n=3).

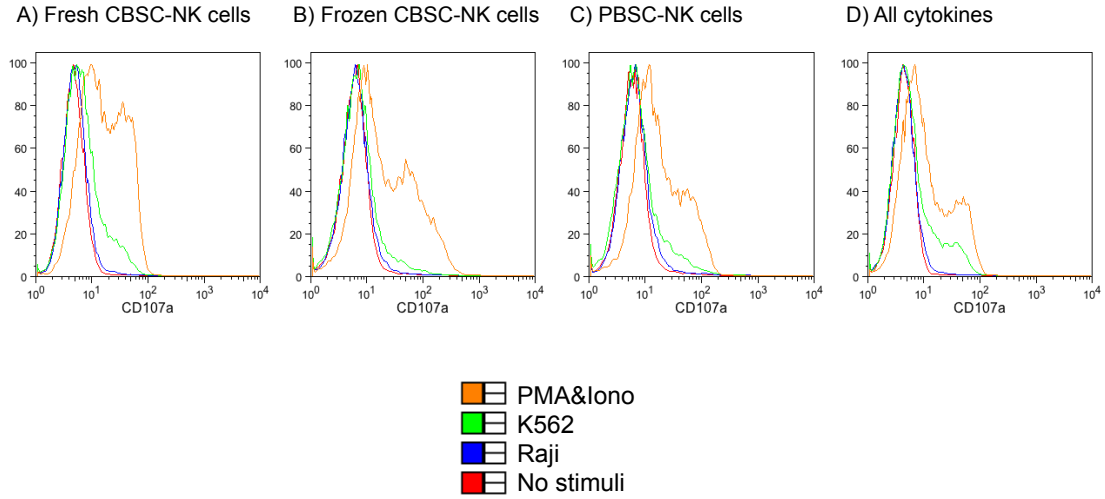


Figure 5.8. Flow cytometry analysis of CD107a. The figure shows representative CD107a histograms of gated $CD56^{+}CD3^{-}$ cells from the lymphocyte gate against PMA&Iono, K562, Raji or without stimuli for A) fresh CBSC-NK cells, B) frozen CBSC-NK cells, PBSC-NK cells and D) CBSC-NK cells using all cytokines.

5.2.3 Natural Killer cells from frozen cord blood stem cell cultures are characterised by low intracellular granzyme B expression but abundant perforin expression

NK cells can lyse virus-infected and tumour cells through activation of granule exocytosis and release of cytotoxic mediators, such as perforin (mediating entry to target cells) and granzymes (initiating apoptosis). The presence of these components in NK cells generated *in vitro* was investigated. Figure 5.9A shows that NK cells from all HSC cultures expressed granzyme B and perforin. Nonetheless, a striking difference in the expression of granzyme B was found; frozen CBSC-NK cells had

significantly less granzyme B expression than fresh CBSC-NK cells and PBSC-NK cells ($p<0.05$). Granzyme B expression in NK cells from fresh CBSC cultures using all cytokines or only IL-15 was similar (figure 5.9B). Moreover, no preferential expression of granzyme B or perforin by a specific NK cell subset according to the surface density expression of CD56 was observed (figure 5.10). Perforin expression was higher in fresh CBSC-NK cells (97.2 ± 0.9%) and PBSC-NK cells (97.9 ± 1.3%) compared to frozen CBSC-NK cells (80.7 ± 14.2%, figure 5.9A), while levels in fresh CBSC-NK cells were similar (96.7 ± 1.6% versus 97.2 ± 0.9%, all cytokines and only IL-15, respectively).

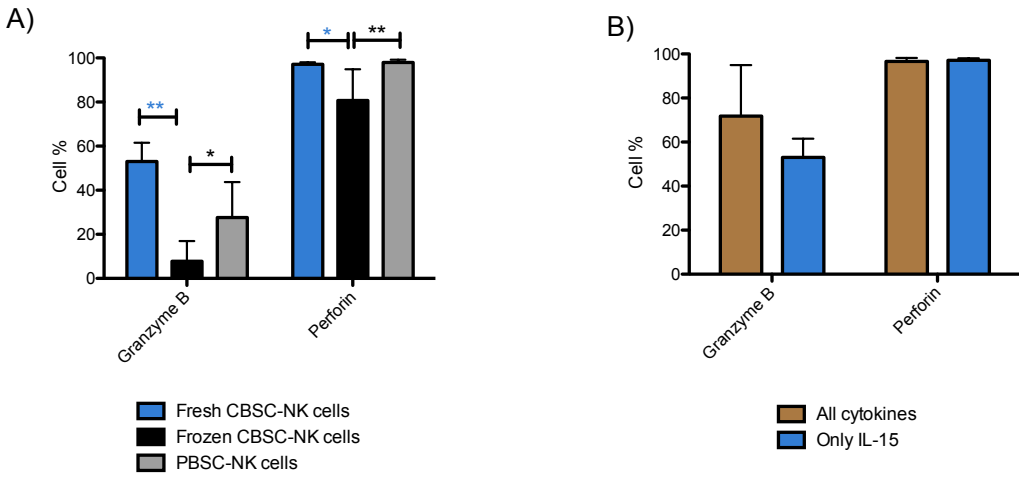


Figure 5.9. Intracellular expression of granzyme B and perforin in NK cells generated *in vitro*. A) Expression of granzyme B and perforin in $CD56^{+}CD3^{-}$ cells from the lymphocyte gate of fresh CBSC cultures (n=3), frozen CBSC cultures (n=9), PBSC cultures (n=6) and B) $CD56^{+}CD3^{-}$ cells from CBSC cultures using all cytokines (n=3) or only IL-15 (n=3). Mann Whitney test was performed. * $p<0.05$, ** $p<0.01$.

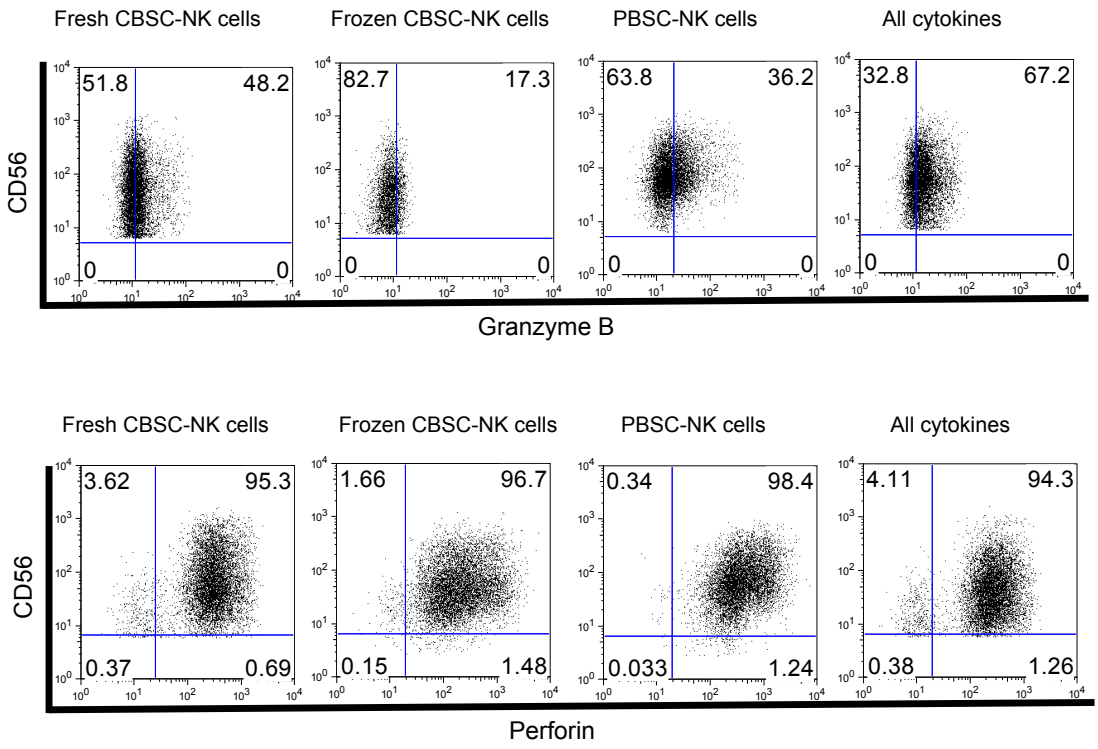


Figure 5.10 Flow cytometry analysis of granzyme B and perforin in NK cells generated *in vitro*. A representative flow cytometry analysis of NK cells from the lymphocyte gate of fresh CBSC, frozen CBSC, PBSC and CBSC cultures using all cytokines for Granzyme B (upper panel) and perforin (bottom panel) is presented.

The low expression levels of granzyme B in NK cells generated *in vitro* led us to determine mRNA levels in these cells. First, granzyme B expression between days 21-35 was screened, as this is when NK cells emerged in HSC cultures. An increase in mRNA expression throughout the weeks was observed, reaching a peak at day 35 (figure 5.11A). Moreover, the comparison of granzyme B mRNA expression at day 35 from NK cells generated *in vitro* to that of resting CB and PB NK cells was performed (figure 5.11B). Although only a trend was observed, it is noteworthy that granzyme B mRNA expression seemed to be higher in NK cells generated *in vitro* compared to CB and PB NK cells.

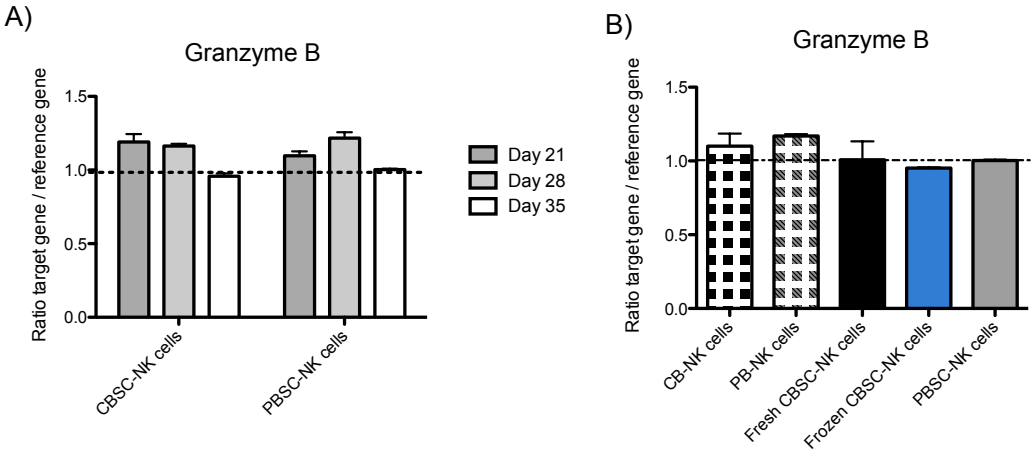


Figure 5.11. mRNA analysis of granzyme B. A) Total RNA was extracted from frozen CBSC-NK cells and PBSC-NK cells harvested at days 21, 28 and 35. B) Total RNA was extracted from resting NK cells from CB (n=3) and PB NK cells (n=3), fresh CBSC (n=3) and frozen CBSC-NK cells (n=4) and PBSC-NK cells (n=3) harvested at day 35. The graphs depict the ratio of granzyme B and the references genes.

In summary, NK cells generated *in vitro* had high expression of perforin, however granzyme B levels were remarkably low in frozen CBSC-NK cells compared to fresh CBSC-NK cells and PBSC-NK cells. Nevertheless, the molecular analysis of granzyme B mRNA revealed that frozen CBSC-NK cells had indeed high expression, comparable to that found in resting CB NK cells and PB NK cells.

5.2.4 ^{51}Cr release assay

5.2.4.1 Killing of K562 in vitro by Natural Killer cells from haematopoietic stem cell cultures

The killing capacity of the generated NK cells using a standard ^{51}Cr release assay with the cell line K562 was investigated. Due to the deficiency of MHC class I in K562 cells, these cells are highly sensitive to NK cell-mediated lysis. K562 cells were labelled with ^{51}Cr and exposed to NK cells generated *in vitro.* Figure 5.12 shows the percentage of specific lysis using different E:T ratios. The percentage of specific lysis observed when using fresh and frozen CBSC-NK cells was very similar at the E:T ratio of 10:1 (41.7 ± 17.2% versus 32.8 ± 3.8% respectively). However, data comparison between frozen CBSC-NK cells and PBSC-NK cells revealed a higher killing capacity by frozen CBSC-NK cells (32.8 ± 3.8% versus 22.4 ± 3.6%; respectively, $p<0.05$). There was no difference in K562 cell lysis by NK cells from fresh CBSC cultures using all cytokines or only IL-15 (figure 5.12B).

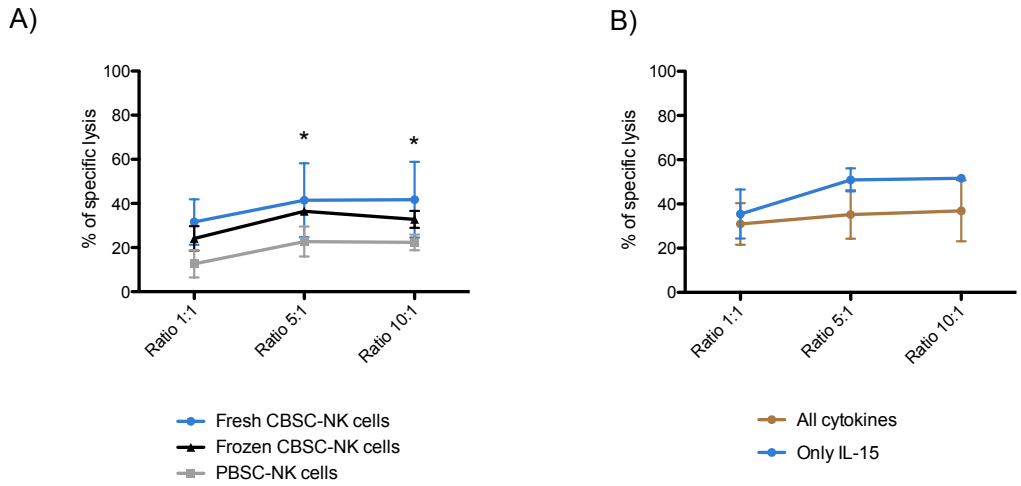


Figure 5.12. ^{51}Cr release assay. A) NK cells from fresh CBSC cultures (n=3), frozen CBSC cultures (n=4), PBSC-NK cells (n=6) and B) NK cells from CBSC cultures using all cytokines (n=3) or only IL-15 (n=3) were co-incubated with ^{51}Cr K562 labelled cells in a standard 4 h ^{51}Cr-release assay. Mann Whitney test was performed. * $p<0.05$.

5.2.4.2 Similar ADCC killing by Natural Killer cells from haematopoietic stem cell cultures

ADCC is an effective mechanism in which CD16 on NK cells recognises the Fc portion of IgG antibodies bound to malignant cells; engagement of CD16 triggers NK cell cytotoxicity against malignant cells. In this experiment anti-CD16 antibodies or an isotype were used to coat murine cell line P815 and used in a standard 4 h ^{51}Cr release assay. We previously described that the expression of CD16 in fresh CBSC-NK cells from either all cytokines or only IL-15 cultures had a tendency for higher CD16 expression (chapter 4, $p=0.0714$); frozen CBSC-NK cells and PBSC-NK cells having a lower expression. Interestingly, no significant differences were found in ADCC activity between NK cells from HSC cultures (figure 5.13A and B). In general, ADCC was low in NK cells from all HSC cultures, with high variability in fresh CBSC samples.

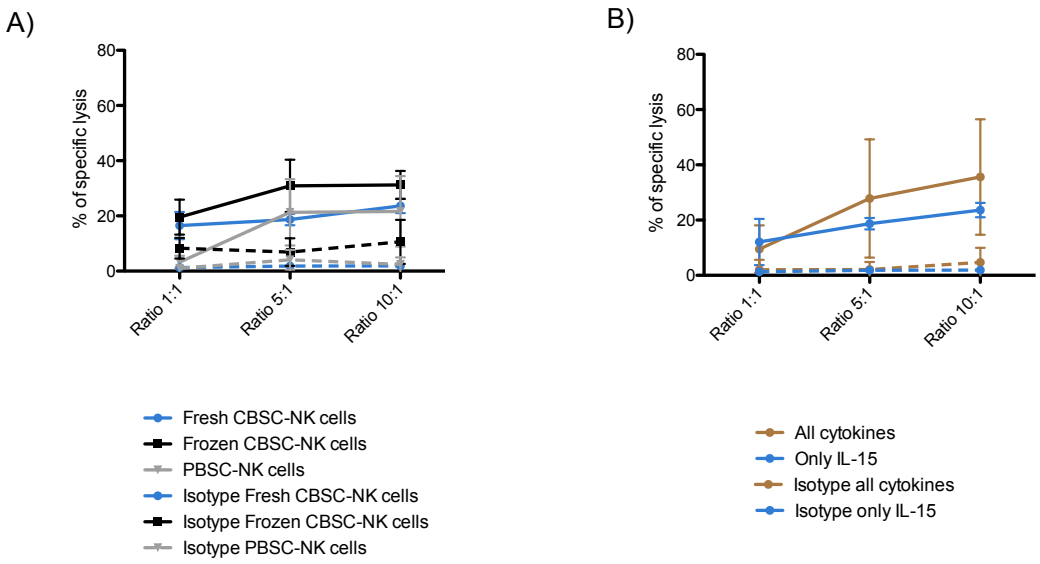


Figure 5.13. Antibody-dependent cell-mediated cytotoxicity. A) NK cells from fresh CBSC (n=3), frozen CBSC cultures (n=8), PBSC-NK cells (n=6) and B) NK cells from CBSC cultures with all cytokines (n=3) or only IL-15 (n=3) were co-incubated with ^{51}Cr labelled P815 cells coated with anti-CD16 or isotype control in a standard 4h ^{51}Cr-release assay. Dotted lines represent the isotype control.

In summary, CBSC-NK cells (fresh and frozen) had similar killing capacity against the cell line K562 and a better killing activity was observed by frozen CBSC-NK cells compared to PBSC-NK cells. ADCC activity did not differ among cultures regardless of the different CD16 expression on NK cells generated *in vitro*.

5.3 Discussion

Many studies have been published describing the basic NK cell killing machinery. We have learned, to a certain extent, how to use these features for immunotherapy purposes. Even though we still have a long way ahead, the current clinical trials have proven than NK cells are safe to infuse with positive effects in the treatment of certain malignancies. Unravelling the functional features of NK cells generated *in vitro* is critical. This will allow us to correlate clinical outcomes with the full characterisation of the infused product. In this chapter we explored the functional potential of NK cells generated *in vitro*. A variety of assays shed light into the remarkable killing capabilities that generated cells *in vitro* have. In particular, frozen CBSC had superior features that enhanced their potential for immunotherapeutic purposes.

During this work, frozen CBSC-NK cells had a superior IFN-γ secretion capacity compared to fresh CBSC-NK cells and PBSC-NK cells. The assessment of IFN-γ in NK cells generated *in vitro* is important due to its potential role in host responses to tumours (Yokoyama *et al.*, 2004) and viral infection (French and Yokoyama, 2004). IFN-γ secretion detected in frozen CBSC-NK cells was similar (PMA&Iono: 4000 pg/ml) to other reports where NK cells derived from CBSC were stimulated with IL-12 and IL-18 (Perez *et al.*, 2006b; Kao *et al.*, 2007); whereas this assay was not performed for PBSC-NK cells (Woll *et al.*, 2005; Giuliani *et al.*, 2008; Woll *et al.*, 2009; Zamai *et al.*, 2012). The intracellular and secreted IFN-γ was very low (3-5%) in NK cells from all HSC cultures when cells were stimulated with K562 cells. The data using freshly isolated CB or PB NK cells also showed very low intracellular IFN-γ expression against K562 cells (Luevano *et al.*, 2012a). Regardless of the difference in secretion of IFN-γ, intracellular IFN-γ remained similar in all HSC cultures when PMA&Iono was used. This indicates that even though the level of intracellular IFN-γ was similar between cultures, frozen CBSC-NK cells were able to produce a vast amount of IFN-γ in response to PMA&Iono. This substantial secretion of IFN-γ is usually associated with the $CD56^{bright}$ subset (Cooper *et al.*, 2001b). This common observation would suggest that CBSC-NK cells resemble this subset. But very interesting data suggests that the $CD56^{dim}$ subset is also able to secrete vast amounts of IFN-γ (De Maria *et al.*, 2011). In contrast to $CD56^{bright}$ cells, $CD56^{dim}$ cells secrete IFN-γ as early as 2 – 4 h after stimulation. $CD56^{bright}$ cells start releasing IFN-γ after 16 h (De Maria *et al.*, 2011). Likewise, during this work NK cells were stimulated for 2 h with PMA&Iono, suggesting that $CD56^{dim}$ cells from frozen CBSC cultures could be responsible for this IFN-γ secretion. This also suggests that fresh CBSC-NK cells and PBSC-NK cells comprise $CD56^{bright}$ cells, which will secrete IFN-γ after 16 h stimulation. A further analysis of the molecular expression

of the IFN-γ mRNA revealed that resting frozen CBSC-NK cells had a tendency for higher IFN-γ mRNA expression compared to resting CB and even PB NK cells. This result is in agreement with a recent study confirming that resting NK cells have high expression of genes encoding effector molecules, like IFN-γ, protease inhibitors and proteases (Bezman *et al.*, 2012a).

Another important factor secreted by NK cells is TNF-α, which can have an anti-tumour effect by downregulating the anti-apoptotic gene bcl-2 in tumour cells (Gillio Tos *et al.*, 1996). TNF-α can also augment NK cell cytotoxicity and response to IL-2 (Owen-Schaub *et al.*, 1988). A moderate secretion of TNF-α when cells were exposed to the cell line K562 was observed, similar to that detected by Cooper and colleagues using $CD56^{bright}$ cells incubated with PMA&Iono (below 300 pg/ml) (Cooper *et al.*, 2001b). Furthermore, CBSC-NK cells showed higher TNF-α secretion when incubation with PMA&Iono was used. This secretion was considerably high (around 900 pg/ml) but far less than that observed by Perez *et al*. using a combination of IL-12 and IL-18 (4000 pg/ml) (Perez *et al.*, 2006b). Although it has commonly been thought that cytokine production and cytolytic functions are not linked, a recent report observed that IFN-γ and TNF-α are functionally linked to their cytolytic activity via upregulation of the adhesion molecule ICAM-1 (Wang *et al.*, 2012). The analysis of ICAM-1 expression on the generated cells was not performed in this study, but NK cells from frozen CBSC cultures certainly have an advantageous IFN-γ and TNF-α secretion profile over fresh CBSC and PBSC cultures that in theory should enhance cytolytic activity.

Besides cytokine secretion, NK cells are able to release cytotoxic granules upon cell activation. Degranulating cells are identified by their surface expression of CD107a. According to some studies, CD107a correlates with cytokine secretion and efficient lysis of target cells (Alter *et al.*, 2004; Penack *et al.*, 2005). The presence of surface CD107a upon exposure of NK cells to K562 or Raji cell lines and PMA&Iono was assessed during this work. The results are similar to the few reports regarding NK cells generated *in vitro* that perform this assay using K562 cell line (20-30%) (Bonanno *et al.*, 2009; Spanholtz *et al.*, 2010; Spanholtz *et al.*, 2011b). In addition, NK cells generated *in vitro* showed degranulation values similar to those exhibited by freshly isolated $CD56^{dim}$ NK cells in CB and PB (Luevano *et al.*, 2012a). As expected, high NK cell degranulation using PMA&Iono (up to 60%) was observed during this work, additionally, it was unexpectedly higher compared to that of $CD56^{dim}$ NK cells in PB (25-35%) (Luevano *et al.*, 2012a). Furthermore, the data showed very low degranulation against Raji cell line, a NK cell-resistant cell line due to the expression of HLA class I. A previous report demonstrated that NK cell priming occurs via CD2

binding to CD15 on tumour cells (Sabry *et al.*, 2011). The absence of CD15 on Raji cells could account for this resistance, however we believe that recognition of HLA by KIRs may also play an inhibitory role. Supporting this statement, a recent study using Raji cells demonstrated that HLA interactions with KIR3DL1 could inhibit NK cell functions (Hasenkamp *et al.*, 2006). The data suggests that a high percentage of NK cells from all HSC cultures were able to degranulate against K562 cells and PMA&Iono and not against Raji cells. Nevertheless, a further characterisation including the screening of KIR3DL1 will provide information on the mechanisms responsible for this inhibition.

NK cell killing can occur via release of granules able to trigger apoptosis of target cells. The presence of granzyme B and perforin in the generated NK cells was investigated during this work using intracellular staining. Granzyme B directly activates Bid, a specific substrate for granzyme B, resulting in caspase activation. Granzyme B depends on being appropriately delivered by perforin. Upon degranulation, perforin inserts itself into the target cell's plasma membrane, forming a pore and allowing granzyme B entry. In fact, a recent report has found that perforin can permeabilise the target cell membrane within an extraordinary short time frame of 30 seconds (Lopez *et al.*, 2013). A higher expression of perforin in NK cells from fresh CBSC and PBSC cultures was found compared to frozen CBSC-NK cells. This high expression of perforin in fresh CBSC-NK cells and PBSC-NK cells was even higher compared to PB $CD56^{dim}$ NK cells. Our results showed a low expression of perforin in $CD56^{bright}$ NK cell subsets from CB and PB and even in CB $CD56^{dim}$ NK cells (Luevano *et al.*, 2012a). The high perforin expression in NK cells generated *in vitro* can be partly explained by the presence of IL-15 in the culture media, reported to upregulate mRNA perforin expression (Gamero *et al.*, 1995).

Conversely, granzyme B expression was significantly lower in frozen CBSC-NK cells (fresh CBSC>PBSC>frozen CBSC). This low expression by frozen CBSC-NK cells resembled that of $CD56^{bright}$ NK cells from CB and PB (Luevano *et al.*, 2012a). Granzyme B greatly contributes to NK cell mediated killing through the activation of caspases directly or indirectly through the mitochondria (Lord *et al.*, 2003). The reason why the intracellular protein was produced in such low amounts (unlike fresh CBSC-NK cells and PBSC-NK cells) remains unknown in our study. The TFs T-BET and EOMES are involved in granzyme B and perforin regulation (Townsend *et al.*, 2004), PBSC and frozen CBSC cultures had a similar T-BET and EOMES mRNA expression (chapter 3) suggesting that these TFs do not account for the low granzyme B expression in frozen CBSC-NK cells. Additionally, high levels of granzyme B mRNA were found in CBSC-

NK cells that could be available for rapid translation and effective killing regardless of the low protein expression. This mRNA expression increased over time and was highly expressed compared to CB NK cells, PB NK cells and NK cells from fresh CBSC and PBSC cultures. Furthermore, it has been reported that rapid translation of perforin and granzyme B messengers occurs when NK cells become activated (White *et al.*). Maybe additional activation signals are needed for granzyme B production in frozen CBSC-NK cells.

In line with other reports showing that *ex-vivo* generated NK cells from CB are highly cytotoxic (Spanholtz *et al.*, 2010; Spanholtz *et al.*, 2011a; Lehmann *et al.*, 2012), NK cells generated in this work also showed high cytotoxicity. Undistinguishable killing capacity was observed when fresh and frozen CBSC-NK cells were used. As IL-15 has been repeatedly reported to augment NK cell cytotoxicity (Decot *et al.*, 2010; Szczepanski *et al.*, 2010), we were expecting to observe a difference between fresh cultures using all cytokines (IL-15: 20 ng/ml) and only IL-15 (50 ng/ml); however, no differences were found. Frozen CBSC-NK cells were significantly better at killing K562 cells than PBSC-NK cells. Moreover, this killing ability without further activation matched that observed by resting PB NK cells (Luevano *et al.*, 2012a) and that observed by Gryzwacz *et al*. in which a mixture of $CD56^{+}CD94^{-}CD117^{high}$ and $CD56^{+}CD94^{+}CD117^{low/-}$ populations was used in a similar assay (Grzywacz *et al.*, 2006). Other studies have reported higher percentages of specific lysis; nonetheless, these studies used longer E:T incubation times (Spanholtz *et al.*, 2010; Spanholtz *et al.*, 2011a) or the addition of cytokines in order to assess NK cell killing (Perez *et al.*, 2006b; Lehmann *et al.*, 2012).

Finally, NK cells are also able to kill via ADCC. Current therapies include the use of ADCC in order to facilitate tumour clearance. For instance, Rituximab is a monoclonal antibody that binds CD20. CD20 is expressed in 95% of B cells in non-Hodgkins lymphomas and not in HSC or healthy cells. NK cells express CD16 (FcγRIIIA); this receptor recognises the Fc portion of antibodies bound to malignant cells. The engagement of CD16 triggers a downstream cascade that elicits NK cell-lysis of the ab-opsinised cell (Campbell and Colonna, 2001). When the Fab portion of Rituximab binds CD20, the Fc portion of this antibody is able to recruit NK cells and therefore mediate ADCC. Bryceson *et al*. highlighted the importance of CD16 in NK cell functions, with CD16 being the only receptor able to elicit a cytotoxic response without the need of co-stimulation (Bryceson *et al.*, 2006b). The ADCC potential in all NK cells generated *in vitro* was assessed using a murine cell line: P815. Due to the higher expression of CD16 in fresh CBSC-NK cells a higher ADCC killing from these NK cells

was expected. Nevertheless, this was not the case, low killing among all HSC cultures without a significant difference between them was observed, although a trend for better killing for frozen CBSC-NK cells was found. It is difficult to compare our results to others using NK cells generated *in vitro*, as we only found one study that includes the ADCC assay (Lehmann *et al.*, 2012) (appendix 3). Additionally, ADCC in CB and PB NK cells was performed; high P815 lysis percentage was found, correlating with the higher CD16 expression on CB and PB NK cells (Luevano *et al.*, 2012a). So in this context, NK cells generated *in vitro* failed to replicate CB and PB ADCC activity. Several studies have now highlighted that CD16 polymorphisms will have an impact on CD16 affinity (Weng and Levy, 2003; Dall'Ozzo *et al.*, 2004). These studies have described a low-affinity FcγRIIIA receptor and a higher-affinity FcγRIIIA receptor according to these polymorphisms (Dall'Ozzo *et al.*, 2004). The polymorphism analysis was not performed during this work. Besides the low expression of CD16 on the generated NK cells, we speculate that the ADCC will also be dependent on the affinity of CD16 conferred by these polymorphisms.

Although testing the generated NK cells against allogeneic targets (K562 or Raji) provides an estimation of the functional capacities of the cells, a more predictive test would include the analysis of the cytotoxicity against fresh autologous tumour cells. These experiments were not performed in our study, but the functional data obtained provides an insight into the cytotoxic capacities of NK cells generated *in vitro*.

The functional characteristics of NK cells generated from HSC cultures comparing fresh versus frozen CBSC and frozen CBSC versus PBSC are shown in figure 5.14 and 5.15, respectively.

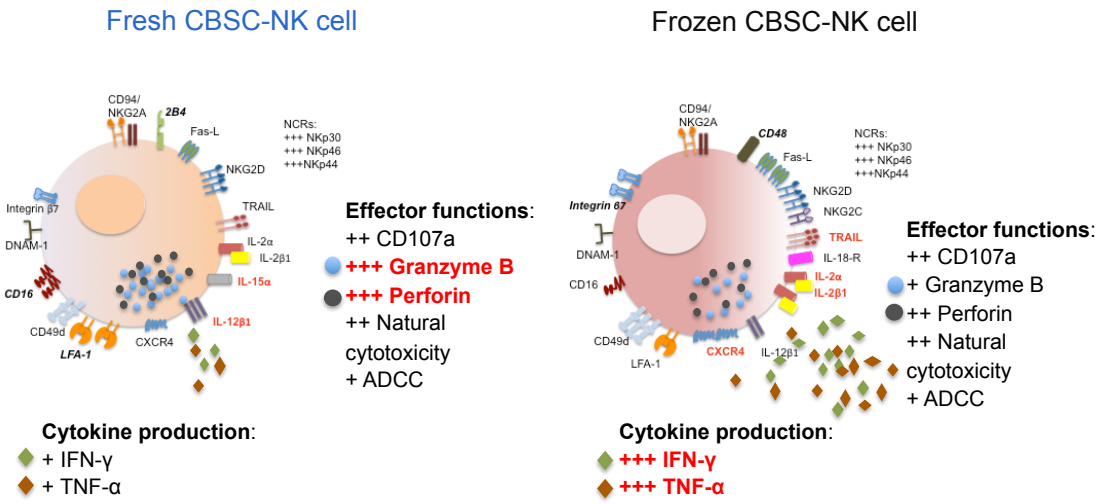


Figure 5.14. Functional characteristics of NK cells derived from CBSC cultures. The figure shows the characterisation of fresh CBSC NK cells and frozen CBSC NK cells. The elements in bold red are significantly higher compared to their counterpart source.

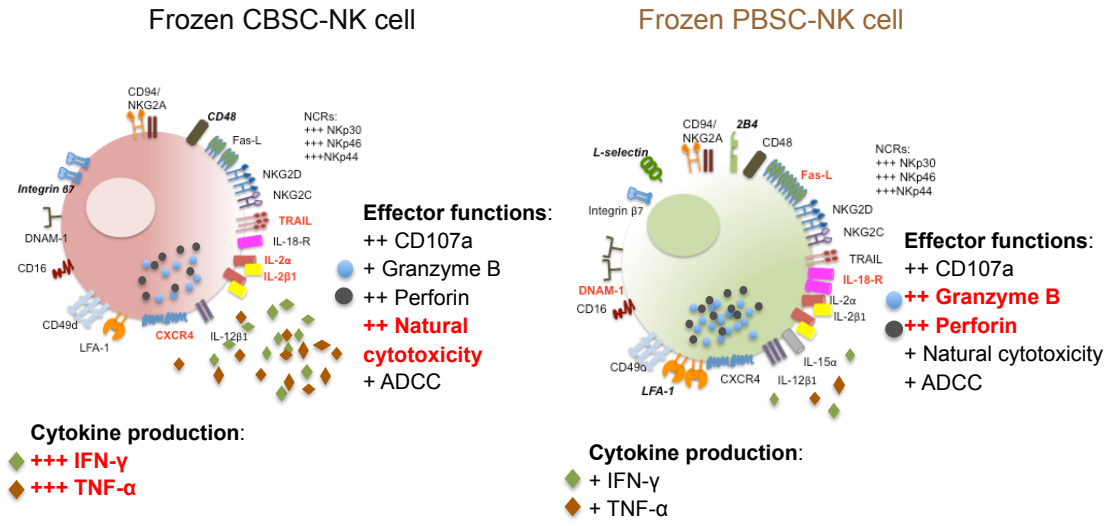


Figure 5.15. Functional characteristics of NK cells derived from frozen CBSC and PBSC cultures. The figure shows the characterisation of frozen CBSC NK cells and frozen PBSC NK cells. The elements in bold red are significantly higher compared to their counterpart HSC source.

Overall, frozen CBSC-NK cells had superior cytokine secretion compared to fresh CBSC-NK cells and PBSC-NK cells, as well as good degranulation capacity accompanied by high levels of intracellular perforin and low granzyme B expression. Nevertheless, high mRNA granzyme B levels were found in the generated NK cells probably accounting for the high cytolytic capacity observed by ^{51}Cr killing assay against cell line K562. The use of only IL-15 for the last three weeks did not impact on any of the features tested. As mentioned before, it could be the case that the use of 20 ng/ml is enough for NK cells to perform their effector functions and the increase of IL-15 concentration (50 ng/ml) will not have an impact on these functions. Altogether, NK cells generated *in vitro* have demonstrated a good cytotoxic capacity along with immunoregulatory potential.

Chapter 6: Role of CD33 expression and effects of IL-12 on Natural Killer cells generated *in vitro*

6.1 Introduction

CD33, a myeloid receptor

Sialic acid-binding Ig-like lectins (siglecs) are proteins involved in signalling and adhesive functions (Crocker *et al.*, 1998). There are two siglec subgroups according to their similarity in intra/extracellular regions: a) the evolutionary conserved ones (siglec-1, -2, 4 and -15) and b) the rapidly evolving CD33-related ones (CD33 [siglec-3], and siglecs 5–11 (Crocker, 2002)). CD33 is commonly known as a myeloid marker expressed by monocytes, neutrophils, dendritic cells, eosinophils, basophils, macrophages and mast cells (Lock *et al.*, 2004). The role of CD33 on myeloid cells has been studied for several years (Paul *et al.*, 2000; Lock *et al.*, 2004). Nevertheless, as CD33 expression is rarely found on lymphocytes, there are few studies regarding its role in these immune cell subsets. NK cell precursors express CD33; however, as development occurs, CD33 expression is lost (Eissens *et al.*, 2012). A study from Hernandez-Caselles found that mitogen- or alloantigen-activated NK cells or T cells could express CD33. This group found a different CD33 mRNA isoform expressed in lymphoid cells and suggested an inhibitory role for this receptor (Hernandez-Caselles *et al.*, 2006).

Because CD33 is expressed in malignancies like AML, it has been used as an immunotherapeutic target. Current approaches include the use of monoclonal antibodies directed against CD33 (Singer *et al.*, 2010; Schubert *et al.*, 2011) or genetically engineered cells specific for CD33 (Marin *et al.*, 2010). For instance, Marin et al. transduced cytokine-induced killer cells with a chimeric receptor encoding CD33 and demonstrated enhanced cytotoxicity against different AML targets although toxicity was observed towards normal haematopoietic $CD34^+$ progenitor cells (Marin *et al.*, 2010). As mentioned in chapter 3, around 50% of NK cells generated *in vitro* during this study expressed CD33. Hence, investigating the role of CD33 expression in NK cells generated *in vitro* is critical. In theory, concomitant anti-CD33 therapies with cell therapy using NK cells generated *in vitro* would likely impact on NK cell functions. Thus, the effect of blocking CD33 on cytolytic and immunoregulatory features on NK cells generated *in vitro* was analysed using a variety of assays.

Modulation of NK cell functions via cytokines; IL-12

Cytokines have been used for decades to activate NK cells and enhance cytotoxicity. Previously, IL-2 was the predominant cytokine used due to promising results observed *in vitro* (Robinson and Morstyn, 1987). Rosenberg and colleagues used autologous lymphokine-activate killer (LAK) cells along with IL-2 infusion in patients with metastatic and advanced cancers, resulting in marked tumour regression in some patients but also toxicity (Rosenberg *et al.*, 1985; Rosenberg *et al.*, 1987). Further studies documented the specific expansion of the immunoregulatory $CD56^{bright}$ cell subset by low doses of IL-2 infusions (Caligiuri *et al.*, 1990; Caligiuri *et al.*, 1993). Moreover, IL-2 acts upon other cells *in vivo* causing severe side effects (Rosenstein *et al.*, 1986; Atkins *et al.*, 1999). In addition, lack of tumour regression as demonstrated by Parkhurst *et al*. has been observed while studying melanoma and renal cell carcinoma (Parkhurst *et al.*, 2011).

Nonetheless, other interleukins have been studied for NK cell activation such as IL-15, IL-21 and IL-12 among others (de Rham *et al.*, 2007; Wendt *et al.*, 2007; Jakobisiak *et al.*, 2011). Our research group has studied the effect of these cytokines either alone or in combination in order to improve CB and PB NK cell cytotoxicity. A promising candidate for NK cell activation *in vitro* is IL-12. This cytokine plays an important role in the regulation of NK cell effector functions (Condiotti and Nagler, 1998) and secretion of IFN-γ (Marcenaro *et al.*, 2005). IL-12 also plays an essential role in the interaction between the innate and the adaptive immune system (Trinchieri, 1995). *In vivo*, phagocytic cells have capacity to produce and secrete IL-12, usually in response to bacteria or intracellular parasites. The signalling pathways of IL-12 in NK cells have been widely studied (Bacon *et al.*, 1995; Lawless *et al.*, 2000; Huang *et al.*, 2011a; Huang *et al.*, 2011b). Additionally, some groups have studied the potential use of IL-12 to enhance NK cell cytotoxicity (Hoshina *et al.*, 1999; Lehmann *et al.*, 2001; Marcenaro *et al.*, 2005) and others have focused on the effect of IL-12 on IFN-γ secretion (Trinchieri, 1995; Lawless *et al.*, 2000). However, the effects of IL-12 on phenotypic and functional features from NK cells generated *in vitro* have not been performed. As a consequence, the present work included assays to assess phenotypic, immunoregulatory and cytolytic features of NK cells generated *in vitro* upon IL-12 incubation. The results from this section will allow manipulation of effector functions of NK cells generated *in vitro* using IL-12.

6.2 Results

6.2.1 Role of CD33 on Natural Killer cells generated *in vitro*

6.2.1.1 Low expression of CD33 expression on cord blood and peripheral blood Natural Killer cells

CD33 is expressed on the surface of normal HPCs (myeloid, erythroid and megakaryocytic) (Andrews *et al.*, 1983) and Kuppfer cells (Rajvanshi *et al.*, 2002). Besides, CD33 has been described as a specific marker for the myeloid lineage (Freeman *et al.*, 1995). In chapter 3 a high expression of $CD45^{+}CD33^{+}$ in all HSC cultures was shown. The role of this marker in NK cells generated *in vitro* was investigated. First, CD33 expression on NK cells from CB and PB was analysed and compared to that of NK cells generated *in vitro*. Figure 6.1 shows the percentage of NK cells that were CD33 positive. Only 4.9 ± 0.9% of CB NK cells and 2.9 ± 0.7% of PB NK cells expressed CD33. However, it was expressed on around 50% of fresh/frozen CBSC-NK cells and PBSC-NK cells. NK cells from all HSC sources had similar CD33 expression without any statistical difference. Thus, the percentage of naturally occurring $CD56^{+}CD33^{+}$ cells is particularly low in blood NK cells.

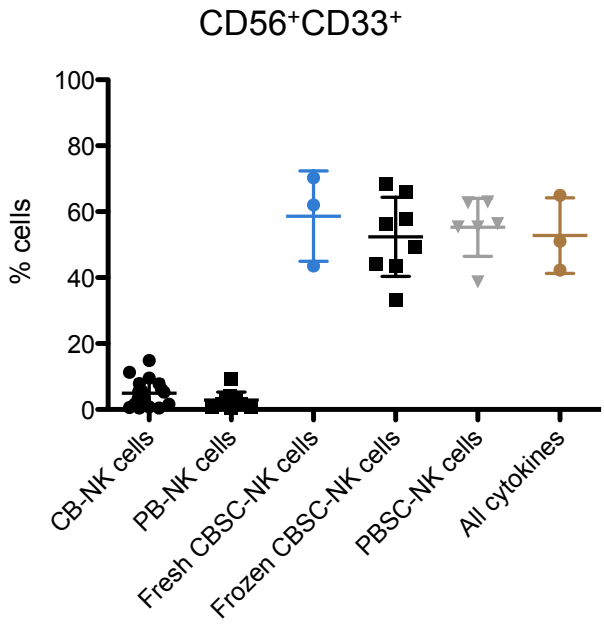


Figure 6.1. CD33 expression on NK cells. Expression of CD33 on $CD56^{+}CD3^{-}$ cells from CB (n=19), PB (n=12), fresh (n=3) and frozen (n=8) CBSC cultures, PBSC (n=6) cultures and CBSC cultures with all cytokines (n=3).

Regarding the expression of CD33 on HSC, it was observed that the majority of $CD34^{+}CD45^{+}$ cells from CBSC and PBSC were positive for CD33 (see section 3.2.5 in chapter 3).

6.2.1.2 Effect of blocking CD33 on cytokine production and secretion by Natural Killer cells generated in vitro

CD33 has two ITIM sequences in its cytoplasmatic domain that convey inhibitory functionality (Crocker, 2005; Hernandez-Caselles *et al.*, 2006). Using a published protocol (Hernandez-Caselles *et al.*, 2006), the effect of blocking CD33 on NK cells generated *in vitro* was investigated. Figure 6.2 depicts intracellular IFN-γ expression of NK cells stimulated with either K562 or PMA&Iono. Blocking CD33 did not impact on IFN-γ expression by NK cells generated *in vitro*.

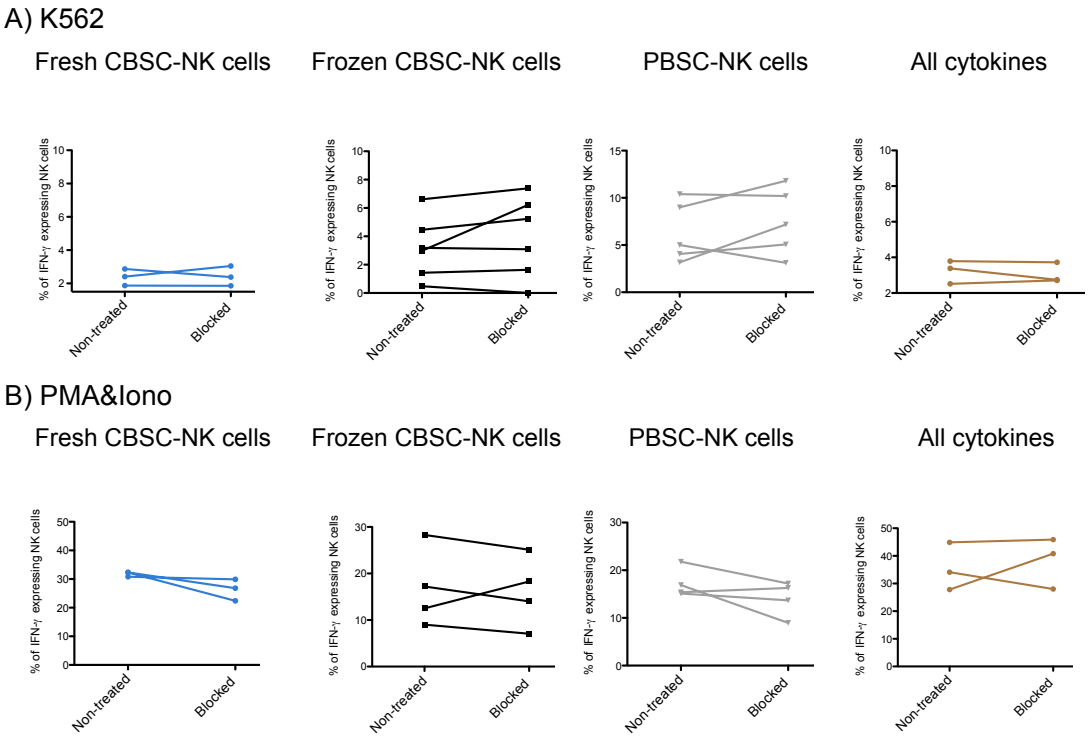


Figure 6.2. **Effect of blocking CD33 in NK cells generated *in vitro* on intracellular IFN-γ expression.** The figure shows IFN-γ production by non-treated and CD33 blocked (blocked) NK cells from fresh and frozen CBSC and PBSC cultures after stimulation with A) K562 or B) PMA&Iono. Paired t-test was performed.

Furthermore, IFN-γ secretion in treated and non-treated cells was analysed (figure 6.3). Surprisingly, IFN-γ secretion against the cell line K562 was increased when CD33 was blocked on fresh and frozen CBSC-NK cells ($p<0.05$). This was not the case for PBSC-NK cells and NK cells from fresh CBSC cultures using all cytokines (K562,

p>0.05). Treated and non-treated NK cells stimulated with PMA&Iono had similar IFN-γ secretion (not significant) (figure 6.3).

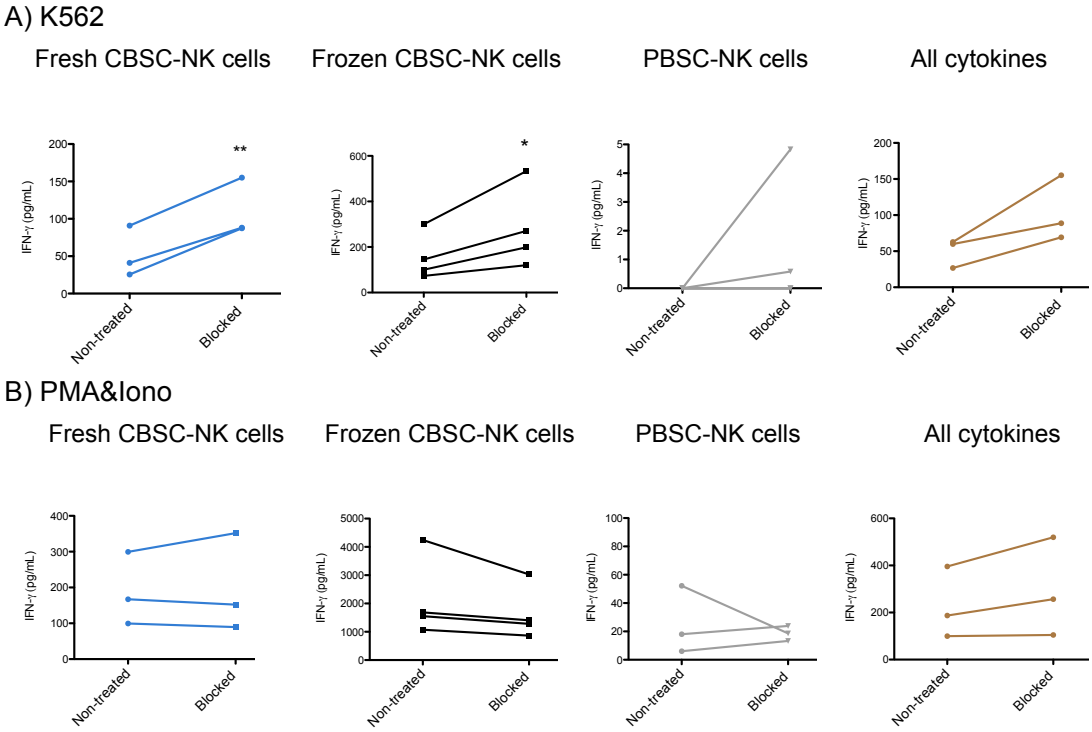


Figure 6.3. Effect of blocking CD33 in NK cells generated *in vitro* on IFN-γ secretion. The figure shows IFN-γ secretion by non-treated and CD33 blocked (blocked) NK cells from fresh and frozen CBSC and PBSC cultures after stimulation with A) K562 or B) PMA&Iono. Paired t-test was performed. * p<0.05, ** p<0.01.

In addition, TNF-α secretion was also investigated in supernatants from NK cells generated *in vitro* after blocking CD33 (figure 6.4). Fresh CBSC-NK cells did not secrete TNF-α when stimulated with the cell line K562, and TNF-α secretion by frozen CBSC-NK cells and PBSC-NK cells was similar among treated and non-treated cells (figure 6.4A). Accordingly, treated and non-treated NK cells from all HSC cultures stimulated with PMA&Iono had similar TNF-α secretion that was not significantly different.

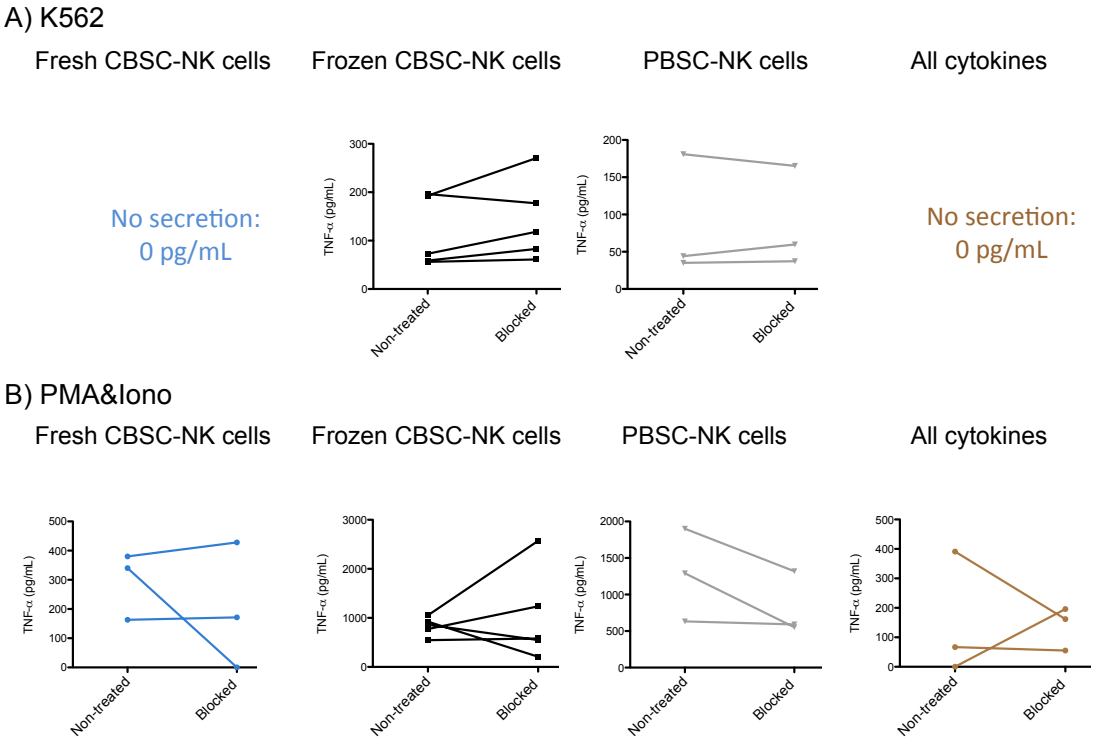


Figure 6.4. **Effect of blocking CD33 in NK cells generated *in vitro* on TNF-α secretion.** The figure shows TNF-α secretion by non-treated and CD33 blocked (blocked) NK cells from fresh and frozen CBSC and PBSC cultures after stimulation with A) K562 or B) PMA&Iono. Paired t-test was performed. * $p<0.05$, ** $p<0.01$.

6.2.1.3 Blocking CD33 does not affect Natural Killer cell degranulation capacity

As described in the previous chapter, CD107a is mobilised to the cell surface following activation-induced granule exocytosis. We decided to investigate if this process would be affected by blocking CD33 on NK cells generated *in vitro*. The results suggest that there is no significant difference between treated and non-treated NK cells from all HSC cultures when cells were stimulated either with the cell line K562 or with PMA&Iono (figure 6.5).

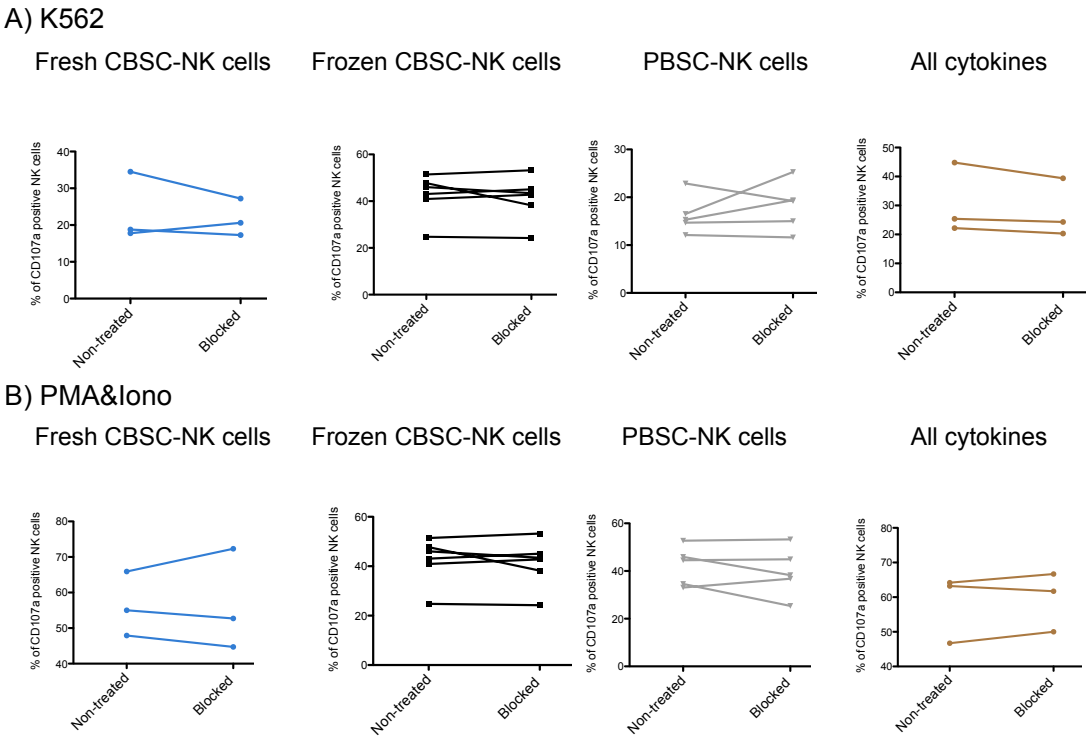


Figure 6.5. Effect of blocking CD33 in NK cells generated *in vitro* on CD107a degranulation. The figure shows degranulation by non-treated and CD33 blocked (blocked) NK cells from fresh and frozen CBSC and PBSC cultures as measured using anti-CD107a antibody after stimulation with A) K562 or B) PMA&Iono. Paired t-test was performed.

6.2.1.4 Blocking CD33 does not impact Natural Killer cell killing

Engagement of CD33 has been shown to inhibit NK cell activity (Hernandez-Caselles *et al.*, 2006). As the majority of NK cells generated during this study expressed CD33, its expression might have clinical implications. The myeloid nature of this receptor and its low expression on healthy cells has made CD33 a therapeutic target. Current therapies include the use of monoclonal antibodies targeting CD33 or engineered killer cells with CD33 specificity. The inhibitory role attributed to CD33 could also be exploited, as blocking of this receptor would in theory enhance NK cell functions. In this section, the role of CD33 was tested in NK cells generated *in vitro*. A standard ^{51}Cr release assay using the cell line K562 and non-treated or CD33-blocked NK cells for all HSC cultures was performed. Figure 6.6 depicts the percentage of specific lysis by treated or non-treated NK cells. No differences in killing of K562 were observed between treated or non-treated cells by NK cells of all HSC cultures.

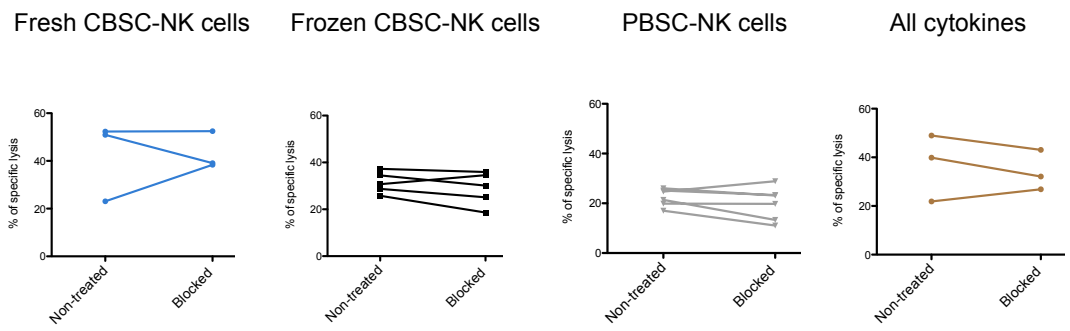


Figure 6.6. Effect of blocking CD33 on K562 killing. The figure shows killing of K562 in a 4 h release assay by non-treated and CD33 blocked (blocked) NK cells from fresh and frozen CBSC and PBSC cultures. Paired t-test was performed.

6.2.2 Impact of IL-12 on Natural Killer cells generated *in vitro*

6.2.2.1 IL-12 incubation allows optimal Natural Killer cell viability

NK cell immunotherapy relies on the presence of NK cells able to perform cytotoxic functions and maintain optimal survival and proliferation. Here, the aim of the study was to enhance the phenotypic and functional features of NK cells generated *in vitro*. NK cell functions can be enhanced by the use of interleukins. A member of the laboratory (Rehab Alnabhan, PhD student) has performed studies highlighting the promising use of IL-12 for NK cell activation. In addition, it is known that IL-12 enhances NK cell cytotoxicity (Naume *et al.*, 1992), secretion of IFN-γ (Kobayashi *et al.*, 1989) and TNF-α (Naume *et al.*, 1992), and increases the expression of adhesion molecules (Rabinowich *et al.*, 1993). Therefore, the effects of IL-12 were evaluated on the generated NK cells; NK cells were harvested at day 35 from CBSC (n=4-9) and PBSC (n=3-6) cultures and incubated with IL-12 (20 ng/ml) for 4, 24 or 40 h. The data generated in the laboratory showed that full NK cell activation (as measured by activating maker expression) was achieved by incubation with IL-12 for 40 h.

First, the effects of IL-12 on NK cell viability were investigated. Living cells translocate phosphatidylserine (PS) from the inner leaflet of the plasma membrane to the outer leaflet in early events of apoptosis. Annexin V preferentially binds to PS, allowing the detection of early apoptotic cells. When combining staining for Annexin V with 7-Amino-actinomycin D (7AAD, binds to double stranded DNA), it is possible to detect cells that underwent apoptosis or necrosis. Thus, live cells would be $AnnexinV^{-}7AAD^{-}$, cells in early apoptotic stages would be $AnnexinV^{+}7AAD^{-}$ and necrotic cells or those in late stage of apoptosis would be $AnnexinV^{+}7AAD^{+}$. Figure 6.7 shows a representative

sample using flow cytometry to assess NK cell viability throughout the incubation time using IL-12.

The percentages of NK cells in early apoptotic stages varied and remained high for CBSC cultures (figure 6.7A). The percentages of NK cells in early apoptotic stages remained stable in PBSC cultures showing lower AnnexinV^{+}7AAD^{-} cells during 0, 4 and 24 h incubation with IL-12. Furthermore, percentages of necrotic cells remained low for all time points and all HSC cultures, ranging from 1-5.9%. This suggests, that the use of IL-12 provides optimal viability for up to 40 h in this study.

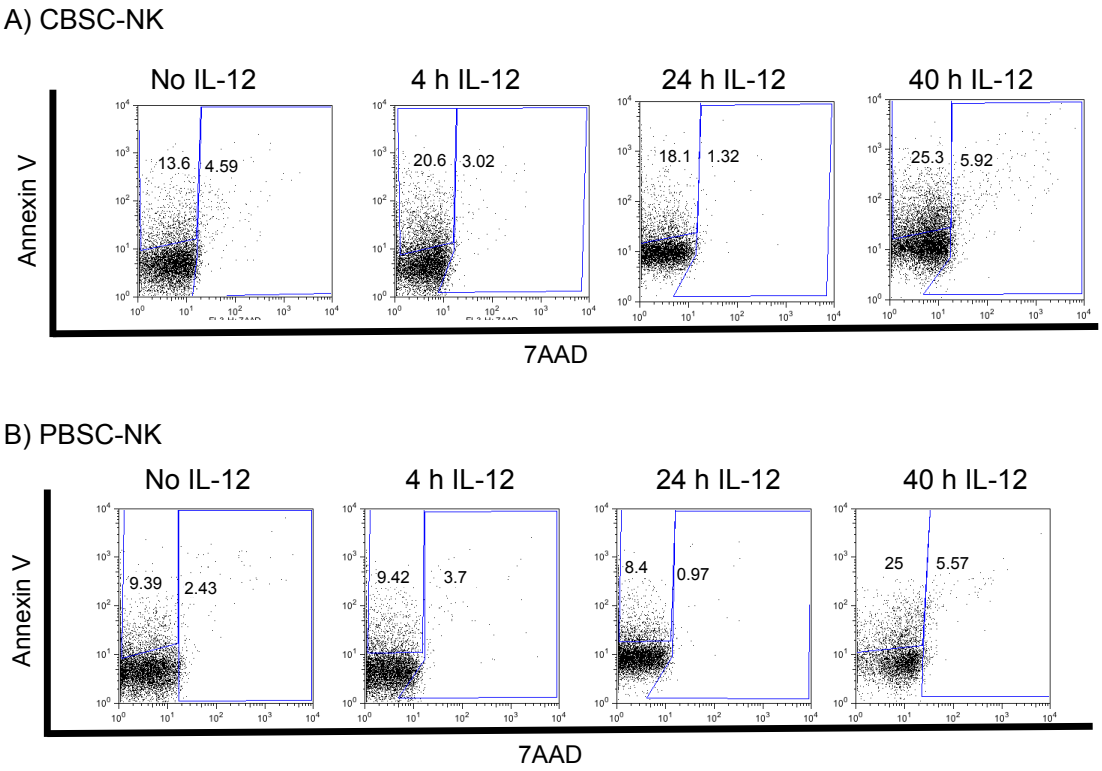


Figure 6.7. Viability of NK cells treated with IL-12. NK cells (percentages come from the CD56^{+}CD3^{-} gate) from A) CBSC and B) PBSC cultures were incubated for 4, 24 or 40 h with IL-12. Viability was measured using Annexin V and 7AAD; the plots show Annexin V versus 7AAD stainings for each time point.

6.2.2.2 IL-12 modifies the repertoire of CBSC-NK cells but not of PBSC-NK cells

NK cell repertoire can be shaped according to the microenvironment and the presence of different interleukins and factors (Di Santo, 2008). Therefore, a full characterisation of NK cells generated *in vitro* and incubated for 40 h with IL-12 was performed. First, the expression of activating and inhibitory markers on the treated cells was analysed. Figure 6.8 shows the expression of activating and inhibitory markers on non–treated NK cells or NK cells incubated for 40 h with IL-12. Unlike PBSC-NK cells (figure 6.8B),

a change of expression of some receptors was observed on CBSC-NK cells treated with IL-12. An increase in CD48 and Fas-L expression and a decrease in NKG2D expression on CBSC-NK cells after 40 h of IL-12 incubation were observed. The expression of inhibitory receptors remained unaltered with IL-12 incubation on NK cells from both HSC cultures.

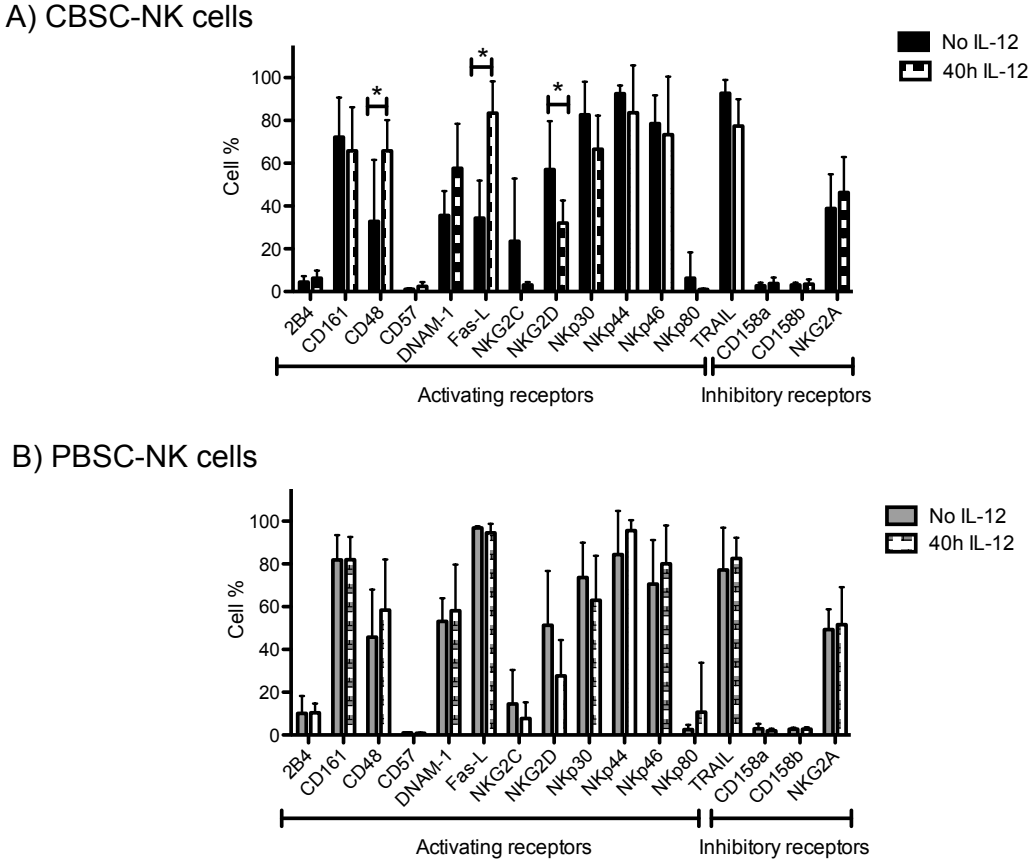


Figure 6.8. Effect of IL-12 on the expression of NK cell activating and inhibitory receptors. NK cells (percentages come from the $CD56^{+}CD3^{-}$ gate) from A) CBSC (n=9) and B) PBSC (n=6) cultures were incubated without stimulation or with IL-12 for 40 h. After incubation, cells were collected, washed and labelled with the appropriate surface antigen or isotype control. Mann-Whitney test was performed, * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

Besides analysing the expression of activating and inhibitory receptors, the impact of IL-12 on the expression of interleukin receptors was studied. Figure 6.9 illustrates the expression of these receptors on CBSC-NK cells and PBSC-NK cells before and after IL-12 incubation. The expression of interleukin receptors on PBSC-NK cells remained unaltered, however IL-12 incubation increased IL-18R and IL-2Rα (CD25) expression on CBSC-NK cells ($p<0.05$).

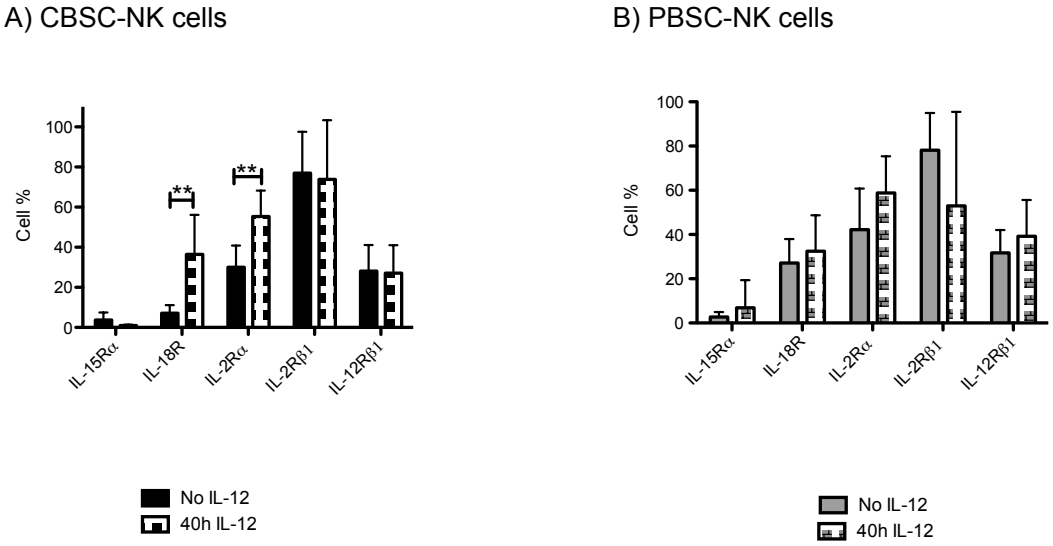


Figure 6.9. **Effect of IL-12 on the expression of interleukin receptor by NK cells.** NK cells (percentages come from the $CD56^{+}CD3^{-}$ gate) from A) CBSC (n=9) and B) PBSC (n=6) cultures were incubated without stimulation or with IL-12 for 40 h. After incubation, cells were collected, washed and labelled with the appropriate surface antigen and isotype control. Mann-Whitney test was performed, * p<0.05, ** p<0.01, ***p<0.001.

The impact of IL-12 on the chemokine receptor repertoire and expression of cell adhesion molecules on NK cells generated *in vitro* was also investigated. The expression of CD49d decreased (p<0.05, figure 6.10A) while LFA-1 expression increased on CBSC-NK cells incubated for 40 h with IL-12 (p<0.05, figure 6.10A). The expression of chemokine receptors on CBSC-NK cells remained unaltered after incubation with IL-12. PBSC-NK cells were not affected by IL-12 incubation, maintaining the same chemokine receptor and adhesion molecules repertoire (figure 6.10B).

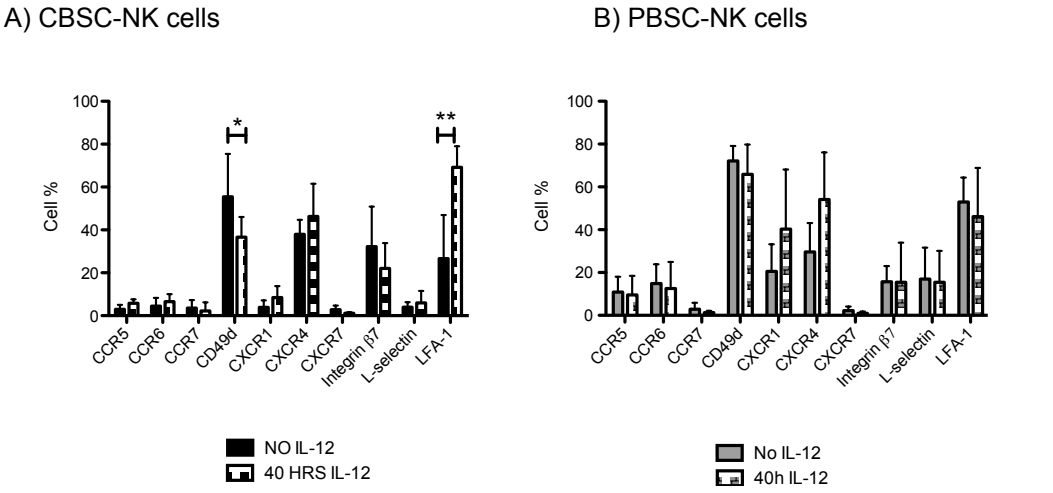


Figure 6.10. Effect of IL-12 on NK cell chemokine receptors and adhesion molecules. NK cells(percentages come from the $CD56^{+}CD3^{-}$ gate) from A) CBSC (n=9) and B) PBSC (n=6) cultures were incubated without stimulation or with IL-12 for 40 h. After incubation, cells were collected, washed and labelled with the appropriate surface antigen and isotype control. Mann-Whitney test was performed, * p<0.05, ** p<0.01, ***p<0.001.

These data suggest that incubation with IL-12 for 40 h did not alter NK cell phenotype of PBSC-NK cells whereas IL-12 modified the phenotype of CBSC-NK cells.

As previously mentioned, CD33 is considered to be a myeloid marker, however, it has also been proposed to be expressed when NK cell activation occurs (Kalberer *et al.*, 2003; Sconocchia *et al.*, 2004; Hernandez-Caselles *et al.*, 2006). The expression of CD33 after incubation with IL-12 was investigated during this study. A gradual increase of CD33 expression on CBSC-NK cells (figure 6.11A) was observed, whereas a decreased expression of CD33 was noted on PBSC-NK cells during the first 24 h incubation with IL-12 (figure 6.11B). Nevertheless, after 40 h incubation with IL-12 PBSC-NK cells showed an increased CD33 expression (figure 6.11B).

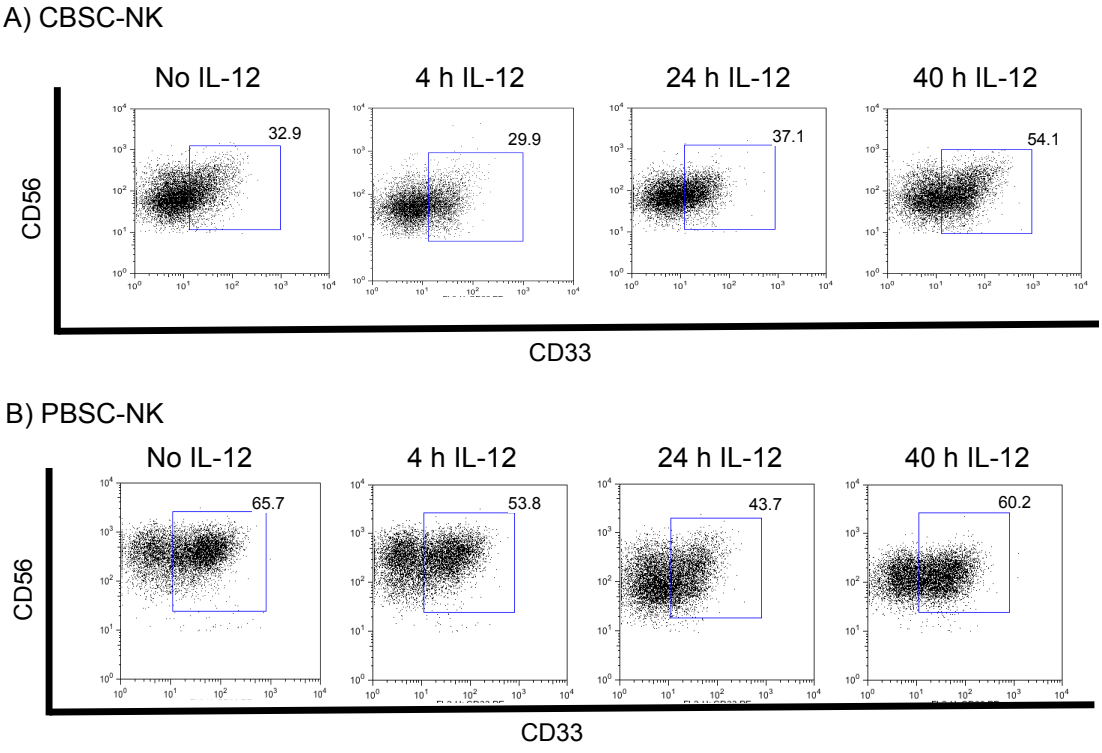


Figure 6.11. Effect of IL-12 on CD33 expression by CBSC-NK cells and PBSC-NK cells. The figure shows a representative sample of $CD56^{+}CD3^{-}$ cells from A) PBSC and B) CBSC cultures incubated without IL-12 or for 4, 24 or 40 h with IL-12. The FACS plots show CD56 versus CD33 expression on NK cells for each time point.

6.2.2.3 IL-12 enhances IFN-γ and TNF-α secretion in CBSC-NK cells

IFN-γ secretion plays an important role during viral infection (French and Yokoyama, 2003) and serves as a bridge for activation of other immune cells (Young and Hardy,

1995; Agaugue *et al.*, 2008). As described in the previous chapter, low intracellular IFN-γ was detected when NK cells were stimulated with K562 cells. In an effort to enhance IFN-γ production, NK cells generated *in vitro* were incubated with IL-12. Frozen CBSC-NK cells had an enhanced IFN-γ expression when they were incubated for 4 and 40 h with IL-12 ($p<0.05$, figure 6.12A). PBSC-NK cells had a gradual increase of intracellular IFN-γ against K562 cells that peaked when 40 h of IL-12 incubation was used ($p<0.05$, figure 6.12A). Intracellular IFN-γ expression was considerably and significantly increased after stimulation with PMA&Iono in CBSC-NK cells incubated for 24 h with IL-12 (figure 6.12B, from 23.1 ± 7.5% to 69.5 ± 18.9% with 24 h incubation with IL-12). Similarly, intracellular IFN-γ expression was also significantly increased in PBSC-NK cells, from 17.7 ± 2.9% to 31.1 ± 7.5% with 40 h incubation with IL-12 (figure 6.12B).

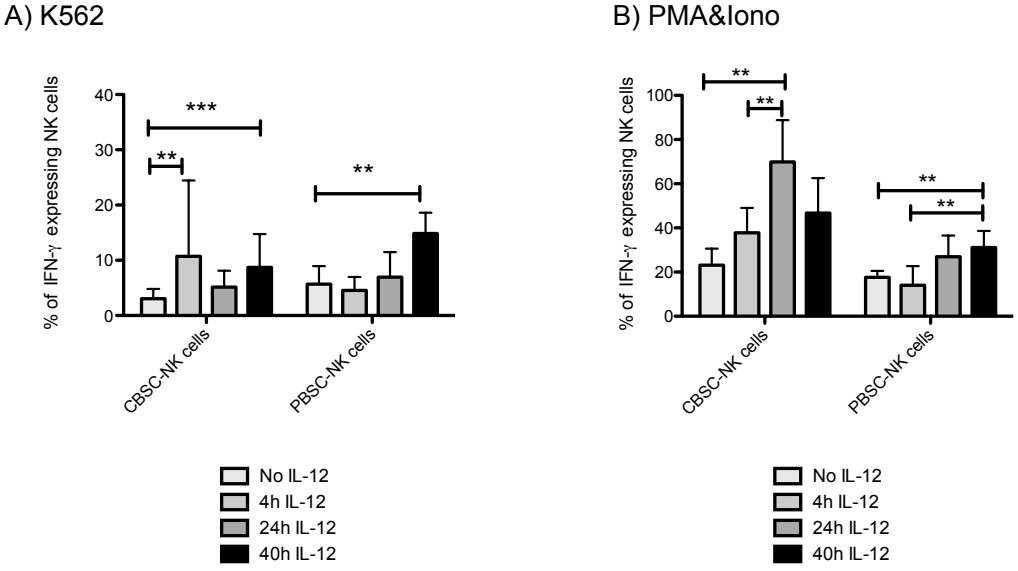


Figure 6.12. Impact of IL-12 incubation on IFN-γ expression of CBSC-NK cells and PBSC-NK cells. Intracellular expression of IFN-γ was analysed by flow cytometry in IL-12 incubated NK cells (percentages come from the $CD56^{+}CD3^{-}$ gate) from CBSC (n=9) and PBSC (n=6) cultures stimulated with A) K562 cells or B) PMA&Iono in the presence of GolgiStop™. Mann-Whitney test was performed, * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

Additionally, the secretion of IFN-γ in NK cell supernatants after incubation with IL-12 was investigated. CBSC-NK cells stimulated with K562 and PMA&Iono secreted high amounts of IFN-γ as described in chapter 5. Pre-incubation with IL-12 for 4 or 24 h dramatically increased the secretion levels of IFN-γ by CBSC-NK cells (K562 and PMA&Iono, figure 6.13A, $p<0.05$), whereas PBSC-NK cells showed a slight increase of IFN-γ secretion but no significant differences were found (K562 and PMA&Iono, figure 6.13B).

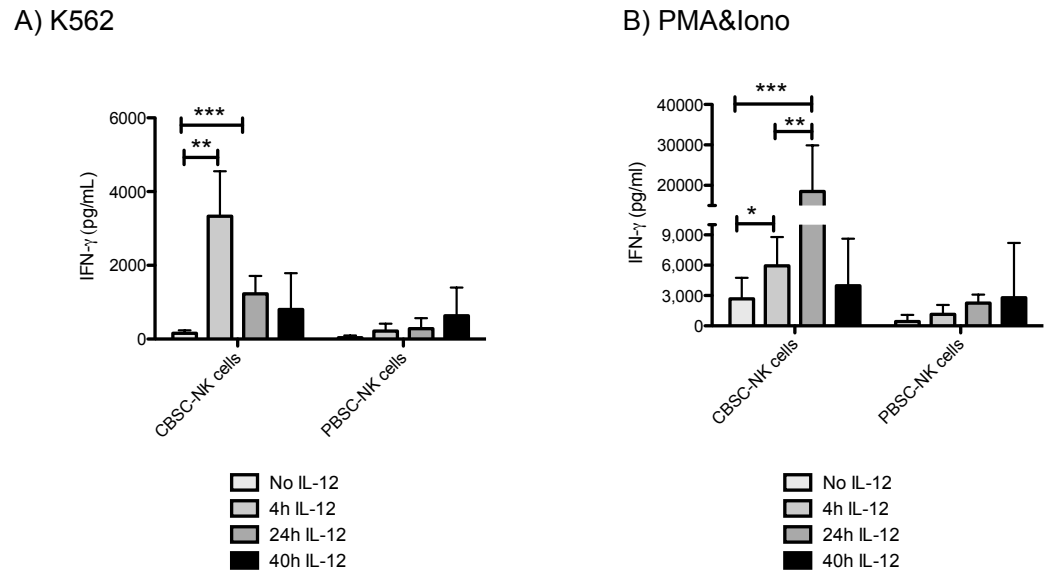


Figure 6.13. Impact of IL-12 incubation on IFN-γ secretion of CBSC-NK cells and PBSC-NK cells. Secretion of IFN-γ was analysed by ELISA in IL-12 incubated NK cells from CBSC (n=9) and PBSC (n=6) cultures stimulated with A) K562 cells or B) PMA&Iono. Mann-Whitney test was performed, * p<0.05, ** p<0.001, ***p<0.001.

Levels of TNF-α were also assessed in supernatants of NK cells incubated with IL-12. When CBSC-NK cells and PBSC-NK cells were stimulated with K562 cells or Raji cells, no differences in TNF-α secretion were found after incubation with IL-12 (figure 6.14A and B). However, when CBSC-NK cells were stimulated with PMA&Iono, an increase in TNF-α secretion was observed when cells were incubated for 24 h with IL-12 (p<0.05). PBSC-NK cells secreted similar amounts of TNF-α regardless of IL-12 incubation (figure 6.14B).

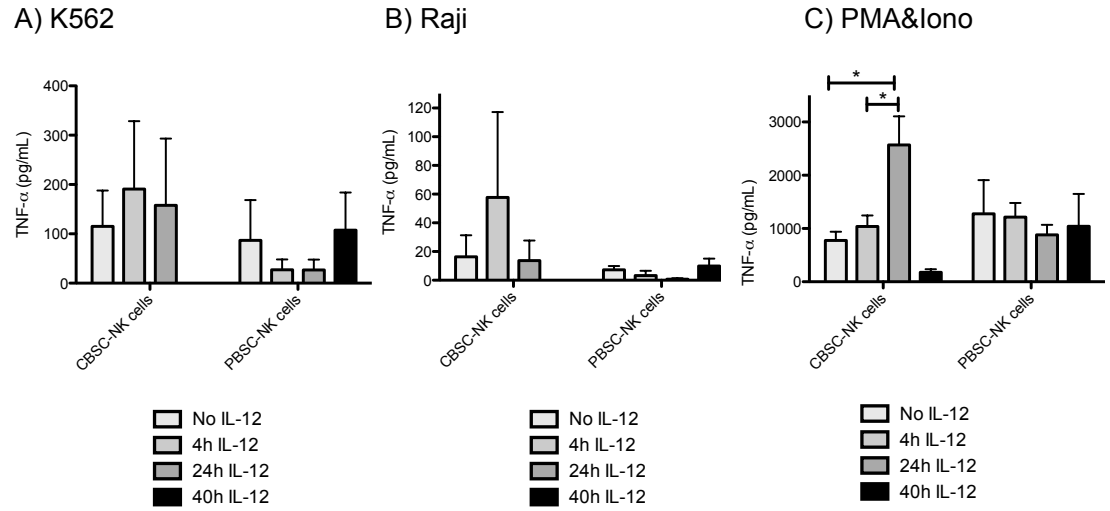


Figure 6.14. Impact of IL-12 incubation on TNF-α secretion by CBSC-NK cells and PBSC-NK cells. Secretion of TNF-α was analysed by ELISA in IL-12 incubated NK cells from CBSC (n=9) and PBSC (n=6) cultures stimulated with A) K562 cells B) RAJI cells or C) PMA&Iono. Mann-Whitney test was performed, * p<0.05, ** p<0.001, ***p<0.001.

Overall, 4 and 24 h incubation with IL-12 enhanced IFN-γ and TNF-α secretion in CBSC-NK cells, whereas the impact of IL-12 on PBSC-NK cell cytokine secretion was subtle and needed 40 h incubation with IL-12.

6.2.2.4 Degranulation of Natural Killer cells from haematopoietic stem cell cultures after incubation with IL-12

IL-12 has been previously used to enhance NK cell cytotoxicity (Marcenaro *et al.*, 2005). In this section, the expression CD107a, used as a degranulation marker as described in chapter 5, was analysed on NK cells generated *in vitro* treated or not with IL-12. Surprisingly, CD107a expression on CBSC-NK cells stimulated with K562 cells decreased after incubation with IL-12, being less significant after 40 h incubation with IL-12 than without ($p<0.05$, figure 6.15A). This was not the case for PBSC-NK cells, where a subtle increase (not statistically significant) was observed with IL-12 incubation (figure 6.15A). CBSC-NK cells and PBSC-NK cells stimulated with PMA&Iono had a similar behaviour, a subtle increase (not statistically significant) was observed with IL-12 incubation (figure 6.15B).

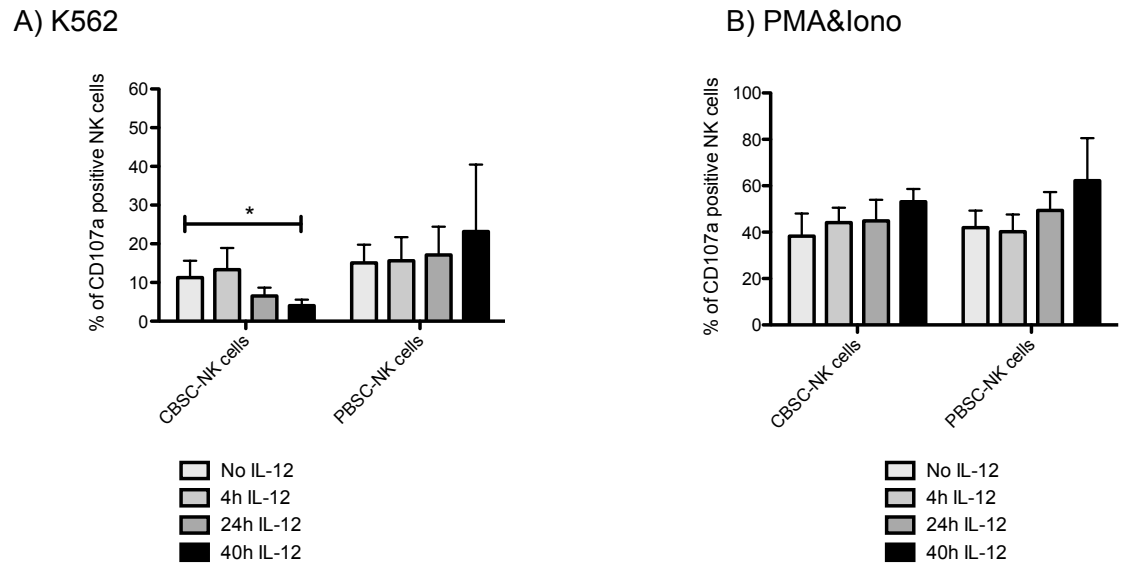


Figure 6.15. Impact of IL-12 incubation on degranulation of CBSC- and PBSC-NK cells. Degranulation was analysed by FACS analysis of surface expression of CD107a in IL-12 incubated NK cells (percentages come from the $CD56^{+}CD3^{-}$ gate) from CBSC (n=9) and PBSC (n=6) cultures stimulated with A) K562 cells or B) PMA&Iono. Mann-Whitney test was performed, * $p<0.05$, ** $p<0.001$, ***$p<0.001$.

6.2.3 IL-12 incubation increases granzyme B expression in CBSC-NK cells and has a minor impact on perforin expression

One of the main concerns while studying the killing machinery of the generated NK cells was the low expression of granzyme B in frozen CBSC-NK cells. It was previously demonstrated that granzyme B mRNA expression was abundant with a higher trend in frozen CBSC-NK cells compared to CB and PB NK cells (chapter 4). Incubation with IL-12 has been shown to increase mRNA levels of granzyme B in human NK cells (Zhang *et al.*, 2008). We investigated whether IL-12 would increase granzyme B expression in NK cells generated *in vitro*. Indeed, as shown in figure 6.16A, incubation with IL-12 for 24 and 40 h significantly increased granzyme B expression (from 7.8 ± 9.2% to 27.5 ± 20.5% with 24 h and 43.6 ± 20.8% with 40 h IL-12 incubation, $p<0.05$). Although there was a slight increase in granzyme B expression using IL-12 incubation in PBSC-NK cells, no significant differences were found between the different IL-12 incubation times (figure 6.16A). Additionally, the intracellular expression of perforin in CBSC-NK cells and PBSC-NK cells incubated with IL-12 was evaluated. The majority of CBSC-NK cells and all PBSC-NK cells had high intracellular perforin expression without IL-12 incubation. However, there was a slight increase in perforin expression in CBSC-NK cells when IL-12 incubations for 4, 24 or 40 h were performed (not statistically significant) while perforin expression in PBSC-NK cells remained unaltered (figure 6.16B).

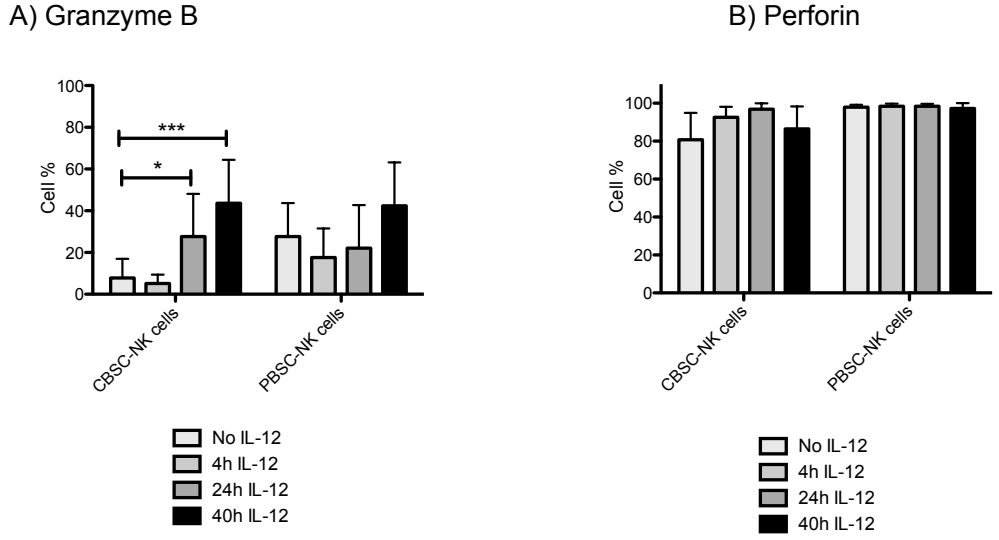


Figure 6.16. Impact of IL-12 incubation on granzyme B and perforin expression from CBSC-NK cells and PBSC-NK cells. Intracellular expression of granzyme B and perforin was analysed by flow cytometry in IL-12 incubated NK cells (percentages come from the $CD56^{+}CD3^{-}$ gate) from CBSC (n=9) and PBSC (n=6) cultures stimulated with A) K562 cells or B) PMA&Iono in the presence of GolgiStop™. Mann-Whitney test was performed, * $p<0.05$, ** $p<0.001$, ***$p<0.001$.

6.2.4 The cytotoxicity of Natural Killer cells generated *in vitro* is enhanced by IL-12 incubation

IL-12 has been previously reported to enhance NK cell cytotoxicity (Naume *et al.*, 1992). First, the expression of one of the most important activating receptors able to trigger NK cell cytotoxicity was followed: CD16. CBSC-NK cells showed a decrease in CD16 expression after 4 and 40 h incubation with IL-12; a peak in CD16 expression was observed when cells were incubated for 24 h with IL-12 (figure 6.17A). As with CBSC-NK cells, CD16 expression on PBSC-NK cells was increased when cells were incubated for 24 h with IL-12 (figure 6.17B). Incubation with IL-12 of PBSC-NK cells for 4 or 40 h did not alter CD16 expression (figure 6.17B).

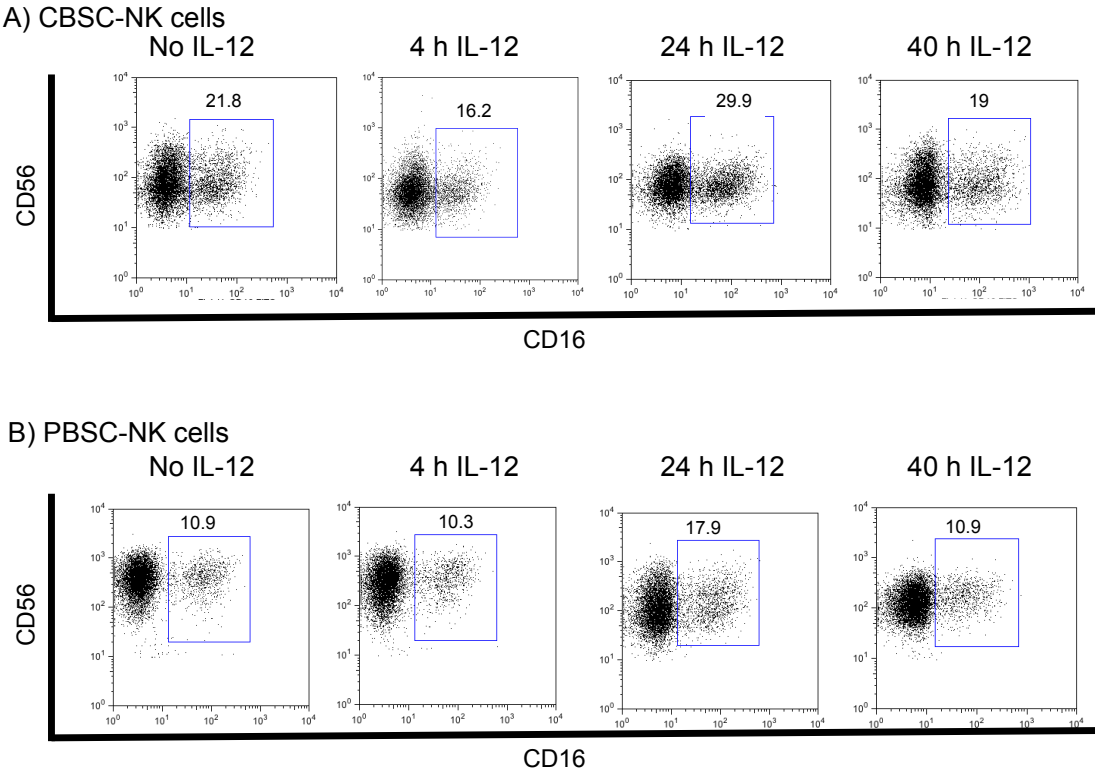


Figure 6.17. Effect of IL-12 on CD16 expression from CBSC-NK cells and PBSC-NK cells. A representative sample of $CD56^{+}CD3^{-}$ cells from A) CBSC and B) PBSC cultures incubated without IL-12 or for 4, 24 or 40 h with IL-12. The plots show CD56 versus CD16 expression on NK cells for each time point.

Next, NK cell cytotoxicity *in vitro* against the cell line K562 was monitored. A standard ^{51}Cr release assay using CBSC-NK cells and PBSC-NK cells generated *in vitro* and incubated with IL-12 was performed. An increase in K562 killing by CBSC-NK cells was observed, although not statistically significant, when cells were incubated for 4 h and 24 h with IL-12. However 40 h IL-12 incubation led to a decline in CBSC-NK cell killing capacity ($p<0.05$, figure 6.18A). PBSC-NK cells showed a better specific lysis with 4 and 24 h IL-12 incubation ($p<0.05$) compared to 40 h IL-12 incubation (figure 6.18A).

ADCC is a major killing mechanism used by NK cells to eliminate malignant or infected cells. According to the CD16 expression pattern on NK cells after IL-12 incubation, both cultures were expected to show a higher ADCC activity when 24 h incubation with IL-12 was used. Indeed, CBSC-NK cells showed a higher ADCC activity when cells were incubated with IL-12 for 24 h (figure 6.18B). In the case of PBSC-NK cells, incubation for 24 h with IL-12 did not show the highest ADCC activity, 40 h of IL-12 incubation demonstrated the best ADCC activity ($p<0.05$, figure 6.18B). The expression of CD16 did not correlate with the observed ADCC activity using 40 h of IL-12 incubation. Conversely, when CBSC-NK cells were incubated for 4 h CD16 expression was low, correlating with the reduced ADCC killing observed (figure 6.18B).

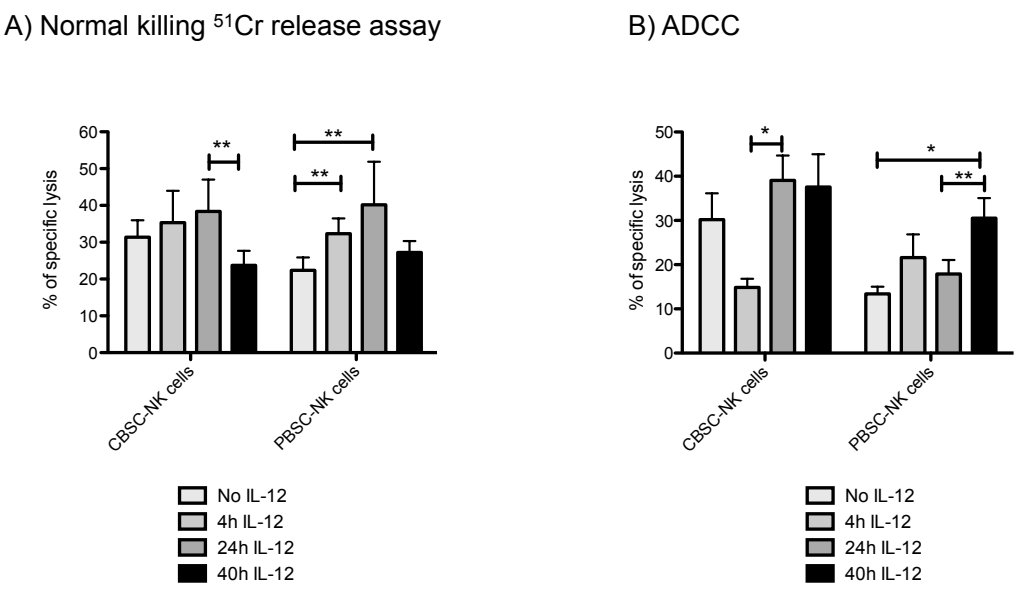


Figure 6.18. Impact of IL-12 incubation on CBSC-NK cells and PBSC-NK cell killing capacity. Killing capacity was analysed in NK cells from CBSC (n=9) and PBSC (n=6) cultures incubated with IL-12 with a standard ^{51}Cr 4 h release assay using labelled A) ^{51}Cr K562 cells or B) ^{51}Cr P815 coated with anti-CD16 or IgG1 isotype (ADCC). Mann-Whitney test was performed, * $p<0.05$, ** $p<0.001$, ***$p<0.001$.

6.3 Discussion

During this work a distinctive phenotype of NK cells generated *in vitro* was observed, including the expression of the myeloid marker CD33. In this chapter the impact of this myeloid marker on the functions of NK cells generated *in vitro* was studied. On the other hand, the potential use of IL-12 in order to enhance phenotypic and functional features of NK cells generated *in vitro* was investigated.

NK cell ontogenesis has been widely studied. The belief that cells have stringent potential to commit to a specific lineage is slowly changing. The findings from separate studies showing that NK cells can be derived from myeloid precursors suggests cell plasticity (Perez *et al.*, 2003; Grzywacz *et al.*, 2011). Taussig *et al*. showed that well-established myeloid markers, such as CD33, CD13, and CD123, are expressed in human long-term repopulating cells from CB and BM (Taussig *et al.*, 2005). Accordingly, the majority of NK cell precursors in our system expressed CD33. Nevertheless, CD33 expression persisted on NK cell precursors during the whole culture and was present in the final NK cell product from all HSC cultures. The search for similar phenotypes ($CD56^+CD33^+$) in blood NK cells revealed the low frequency of these cells in CB and PB. Indeed, CD33 is usually only expressed in myeloid cells and not present in healthy lymphocytes (Tanimoto *et al.*, 1989). Others also found low frequency of $CD33^+$ NK cells in PB or CB (Handgretinger *et al.*, 1993; Hernandez-Caselles *et al.*, 2006). In fact, due to this specific expression, therapies using anti-CD33 monoclonal antibodies (Lintuzumab or Gemtuzumab Ozogamicin [Mylotarg]) were previously used for the treatment of AML (Sievers *et al.*, 1999; Raza *et al.*, 2009). These treatments were abandoned in 2010 since they failed to provide benefits over other conventional therapies (Feldman *et al.*, 2005; FDA, 2010). Nevertheless, other therapies using CD33 are currently studied, such as the use of dual-targeting antibodies (CD123/CD33 (Kugler *et al.*, 2010) or CD19/CD33 (Schubert *et al.*, 2011)) for AML or mixed leukaemia treatments. Although these studies are in pre-clinical phases, anti-CD33 therapies should in theory be administered prior to NK cell immunotherapy. Although not studied in this work, the concomitant administration of such anti-CD33 antibody therapies with NK cells generated *in vitro* would definitely have an impact on NK cells expressing CD33.

A recent study on NK cell development using BM and CB precursors by Eissens *et al*. found that CD33 was only expressed from stages 2-3 (Eissens *et al.*, 2012). To our knowledge, there is only one study reporting expression of CD33 on NK cells generated *in vitro* (Vitale *et al.*, 2008). Vitale *et al*. observed the appearance of a

$CD33^+$ population with myelomonocytic features as shown by forward scatter (FSC) and side scatter (SSC) parameters (Vitale *et al.*, 2008). Conversely, NK cells generated *in vitro* in our study expressed CD33 but were within the lymphocyte gate and lacked myelomonocytic features as shown by FSC and SSC (data not shown). From these data several questions arise. What is the role of CD33 during NK cell development? What are the consequences of CD33 expression on NK cells generated *in vitro*? Apparently CD33 is dispensable for NK cells, as CB or PB NK cells have very low or undetectable expression (Handgretinger *et al.*, 1993; Hernandez-Caselles *et al.*, 2006). But, what about the few NK cells that are expressing CD33 in CB or PB? Does CD33 expression confer different cytotoxic properties? Since CD33 is mostly considered a myeloid marker, few studies have addressed the role of CD33 in lymphocytes (Hernandez-Caselles *et al.*, 2006). CD33 has a putative function in cell adhesion through sialic acid cell binding (Ulyanova *et al.*, 1999); moreover, due to its ITIM motif it may be implicated in cellular activity inhibition (Taylor *et al.*, 1999) via src homology region 2-containing protein tyrosine phosphatase-1 (SHP-1) and SHP-2 (Paul *et al.*, 2000). Actually, previous reports suggested an inhibitory role of CD33 on myeloid cells (Paul *et al.*, 2000) and recently in NK cells derived from CB $CD34^+$ (Handgretinger *et al.*, 1993) or from NK cell lines (Hernandez-Caselles *et al.*, 2006). With this in mind, we decided to investigate if CD33 expression would have a similar effect on effector functions of NK cells generated *in vitro*.

It is important to consider that FACS-sorting was not performed during this study, thus each assay comprises a mixture of $CD56^+CD33^+$ and $CD56^+CD33^-$ cells. In the previous chapter it was reported that intracellular IFN-γ in NK cells from all HSC cultures was very similar but secretion was higher in frozen CBSC-NK cells. After treatment with anti-CD33, no effect on intracellular IFN-γ expression was observed in NK cells from CBSC or PBSC cultures stimulated either with K562 cells or PMA&Iono. Similarly, blocking of CD33 did not affect secretion of IFN-γ in NK cells stimulated with PMA&Iono. Nevertheless, a difference in secreted IFN-γ by NK cells from fresh and frozen CBSC-NK cells was observed when stimulation with K562 was performed. When CD33 was blocked, IFN-γ secretion by NK cells was increased. CD33 ITIM motif recruits SHP-1 and SHP-2 (Paul *et al.*, 2000). Working with NK cell lines, Purdy *et al*. found that SHP-2 suppresses cytolytic activity and IFN-γ secretion (Purdy and Campbell, 2009). Moreover, Handgretmger *et al*. described that about 8% of CB NK cells express CD33 and these cells exhibit low cytotoxicity against cell lines such as K562 in comparison to $CD56^+CD33^-$ cells (Handgretinger *et al.*, 1993). Maybe blocking CD33 on NK cells generated *in vitro* inhibited the phosphorylation of SHP-1 and SHP-2, thus preventing the suppression of IFN-γ secretion. Regarding the cell adhesion role

of CD33, the study from Freeman *et al*. revealed that the K562 cell line showed little binding to CD33 in a solid-phase binding assay with Fc-CD33 (Freeman *et al.*, 1995). In fact, according to this study myelomonocytic-like and promonocytic-like cells express CD33 ligands; these cells are found in the BM. The interactions of NK cells generated *in vitro* with these cells were not investigated in this study. It would be interesting to study these interactions as NK cells generated *in vitro* hold the potential to migrate to the BM due to CXCR4 expression, where myeloid and lymphoid development occurs. Figure 6.19 shows a possible scenario in which binding of CD33 and ligands in K562 cells occurs acting as an inhibitory receptor, although further investigations should be performed to pinpoint potential ligands and signalling pathways. In this context, administration of anti-CD33 in the treatment of AML would actually enhance NK cell ability to secrete IFN-γ. This enhancement would only be effective for IFN-γ secretion. Regardless of the use of K562 or PMA&Iono stimulation on NK cells generated *in vitro*, we did not observe an alteration of TNF-α secretion, degranulation or killing capacity when CD33 receptor was blocked. This could be attributed in part to CD33 internalisation, as some studies reported rapid CD33 internalisation after antibody binding (van Der Velden *et al.*, 2001; Walter *et al.*, 2008).

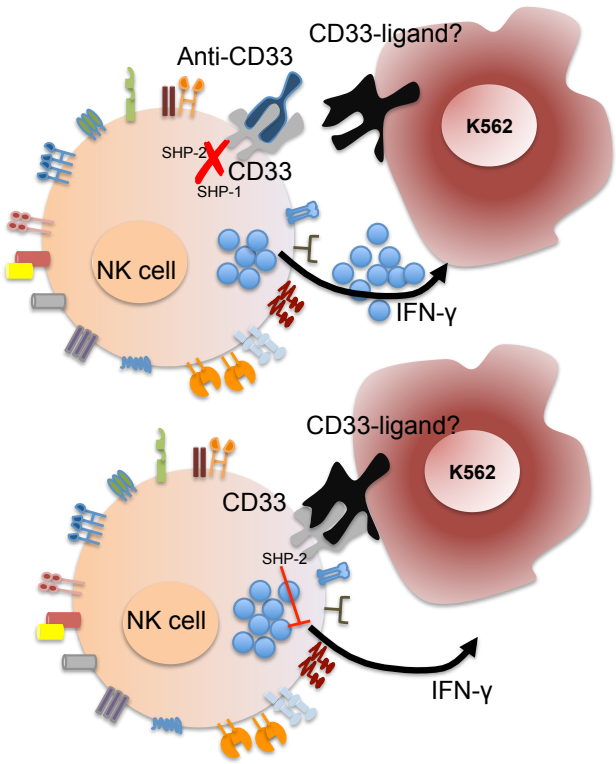


Figure 6.19. Possible inhibitory role of CD33 on NK cells. The upper panel depicts the interaction between a treated NK cell (CD33 blocked) and a K562 cell. The bottom panel shows the interaction of a non-treated NK cell with a K562 cell.

During the second section of this chapter we studied the effects of IL-12 on the functions of NK cells generated *in vitro*. Previous studies in our laboratory have shown

that IL-12 is able to activate PB and CB NK cells (Rehab Alnabhan), 40 h being the optimal incubation time. We first investigated whether NK cell viability would be affected by IL-12 incubation. A study by Huang *et al*. reported that NK cell exposure to IL-12 resulted in cell activation but prolonged exposure induced apoptosis (Huang *et al.*, 2011b). In this study, the use of IL-2 and of 40 ng/ml of IL-12 for 48 h resulted in 50% apoptosis of NK cells. A direct comparison with our study cannot be performed as we used 20 ng/ml of IL-12 and a maximum of 40 h of incubation. However, dead cell percentages in our cultures were lower (maximum 5.92%). Therefore, in our hands, viability of NK cells during IL-12 incubation was barely affected.

Since viability remained unaltered, the phenotypic changes that IL-12 could induce on NK cells generated *in vitro* were studied. Regardless of the abundant literature studying IL-12 effects on NK cell functions and signalling pathways, few studies performed a phenotypic NK cell characterisation (Rabinowich *et al.*, 1993; Loza and Perussia, 2004b). In our hands, incubation with IL-12 did not modify the repertoire of PBSC-NK cells: activating receptors, inhibitory receptors, adhesion molecules, chemokine receptors and interleukin receptor expression remained unaltered. In contrast, IL-12 incubation of CBSC-NK cells modified the expression of some activating receptors (CD48, Fas-L and NKG2D), interleukin receptors (IL18R and IL-2Rα) and adhesion molecules (CD49d and LFA-1). In an effort to study a $CD56^{+high}$ cell subset in PB, Loza *et al*. found that this cell subset represents IL-12 activated cells *in vivo* (Loza and Perussia, 2004b). During the characterisation of this $CD56^{+high}$ cell subset, an increased 2B4 expression and decreased NKp44 expression after IL-12 incubation was found (Loza and Perussia, 2004b). We did not observe an increased expression of these markers on CBSC-NK cells or PBSC-NK cells during our study; but CD48 and Fas-L expression was higher on CBSC-NK cells when 40 h of IL-12 incubation was used. We previously discussed the low expression of Fas-L in CB and PB NK cells and the potential advantages of Fas-L expression on NK cells generated *in vitro* in chapter 4. The use of IL-12 increased Fas-L expression of CBSC-NK cells to similar values observed in PBSC-NK cells. Again, this expression could be translated into a better killing potential of NK cells generated *in vitro.* The higher CD48 expression would also have a positive impact, since interactions between 2B4 and CD48 are known to activate NK cells (Messmer *et al.*, 2006). In accordance with our results, incubation of PB NK cells with IL-12 had no effect on CD49d expression but increased the expression of LFA-1 (Rabinowich *et al.*, 1993), which would confer adhesion and cell activation advantages. Similarly to our results in CBSC-NK cells incubated with IL-12, it has been reported that IL-12 up-regulated IL-2Rα in PB NK cells (Naume *et al.*, 1992) and CB MNCs (Condiotti and Nagler, 1998) suggesting better proliferation capacity. IL-

12 also increased the expression of IL-18R in CBSC-NK cells; Lawless *et al*. reported a similar outcome while studying the signalling pathway of IL-12 (Lawless *et al.*, 2000). IL-12 signalling involves activation of STAT4, which in turn has been reported to increase the expression of IL-18R (Lawless *et al.*, 2000). On the other hand, IL-12 has been reported to increase L-selectin expression (Loza and Perussia, 2004b); yet, our data did not corroborate this observation. The downside of the effect of IL-12 on CBSC-NK cells was the reduction of NKG2D expression; an important receptor involved in recognition of transformed cells (Burgess *et al.*, 2008). Another interesting fact was the increased CD33 expression observed in CBSC-NK cells with IL-12. Hernandez-Caselles *et al*. suggest that CD33 expression can be induced on activated NK cells (IL-2 and allogeneic stimuli) (Hernandez-Caselles *et al.*, 2006). We used IL-12, although these cytokines do not clearly share a signalling pathway (IL-2 activates JAK1 AND JAK3 (Yu and Burakoff, 1997) and IL-12 JAK2 and TYK2 (Watford *et al.*, 2003)), perhaps there is a common intermediary that regulates CD33 expression. At this point, our data suggested that CBSC-NK cells could have a better killing potential upon IL-12 incubation due to the modification of their repertoire compared to PBSC-NK cells.

Although not studied in this work, IL-12 is able to activate different pathways, among these, the Jak-signals and activator of TFs (STATs). STAT4 might be one of the most important, playing a key role in the secretion of IFN-γ (Bacon *et al.*, 1995). Indeed, intracellular IFN-γ expression and secretion were drastically improved in CBSC-NK cells upon IL-12 activation. It was interesting to observe that depending on the stimulation source, IFN-γ would be differentially expressed/secreted. When cells were stimulated with K562, 4 h incubation with IL-12 showed the highest expression/secretion in CBSC-NK cells, while the effects using PMA&Iono were clearly higher when 24 h incubation with IL-12 was performed. Similar results for TNF-α were observed in CBSC-NK cells using PMA&Iono. It was also evident that PBSC-NK cells had a different response upon IL-12 activation regardless of the similar IL-12Rβ1 expression in CBSC-NK cells (chapter 4). Only intracellular IFN-γ expression increased upon IL-12 incubation (peak at 40 h), whereas the secretion of IFN-γ and TNF-α did not change. In line with our observations, low levels of TNF-α were produced in PB NK cells when cells were treated with IL-12 (Naume *et al.*, 1992).

The killing capacity of CBSC-NK cells and PBSC-NK cells upon IL-12 activation using several assays was also studied. CBSC-NK cells stimulated with K562 showed decreased CD107a detection after IL-12 incubation, however CD07a expression was not altered when PMA&Iono was used. Therefore, according to our study, CD107a

expression may not accurately correlate with NK cell cytokine secretion. Next, the expression of granzyme B and perforin upon IL-12 incubation was studied. One of the major concerns discussed in chapter 4 was the absence of granzyme B in frozen CBSC-NK cells, although high mRNA levels were found. In fact, IL-12 incubation of frozen CBSC-NK cells considerably increased granzyme B expression to a level comparable to that of PBSC-NK cells. This data is in accordance with a previous study reporting mRNA granzyme B and perforin upregulation by IL-12 (Salcedo *et al.*, 1993). The majority of the generated cells expressed perforin even without IL-12 incubation, thus further incubation with IL-12 did not have a major impact on perforin expression. These data suggest that NK cells generated *in vitro* were equipped for NK cell cytolytic functions. In our study, 24 and 40 h of IL-12 incubation provided CBSC-NK cells with intracellular granzyme B. Similarly, Marcenaro *et al*. showed that NK cells incubated overnight with IL-12 displayed strong cytotoxicity against various tumour cell lines (Marcenaro *et al.*, 2005). In fact, 24 h incubation showed the highest K562 lysis by CBSC-NK cells and PBSC-NK cells, while 40 h caused a decrease in K562 lysis in both cultures. Another report showed enhanced NK cell cytotoxicity even with incubation with IL-12 for 3 days (Naume *et al.*, 1992).

Finally, the impact of IL-12 on ADCC capacity of the generated cells was studied. The expression of CD16 upon IL-12 incubation was first analysed. A peak in CD16 expression was observed on CBSC-NK cells and PBSC-NK cells after 24 h incubation with IL-12, while incubation for 40 h led to a decline in CD16 expression. During this work the data regarding CD16 expression and IL-12 activation showed no correlation. One study reported that exposure of PB NK cells to IL-12 decreases CD16 expression (Loza and Perussia, 2004b), although a 5-day incubation was performed in this study. Another study using PB and CB MNCs found that incubation for 18 h with IL-12 did not alter CD16 expression (Condiotti and Nagler, 1998). Although metalloproteinases have been involved in the shedding of CD16 (Grzywacz *et al.*, 2007; Romee *et al.*, 2013), this phenomenon was not studied in our system however shedding of CD16 cannot be ruled out.

The results from the previous chapter showed low ADCC potential from NK cells generated *in vitro*. CB NK cell cytotoxicity via ADCC can be enhanced with IL-12 according to a previous study (Nguyen *et al.*, 1998). Even though a high expression of CD16 was found when CBSC-NK cells and PBSC-NK cells were incubated for 24 h with IL-12, the highest ADCC killing was not observed at this time point. PBSC-NK cells showed a higher ADCC killing with 40 h incubation with IL-12, and CBSC-NK cells had similar ADCC killing after 0, 24 and 40 h incubation with IL-12. Nguyen *et al*.

suggested that the enhanced ADCC activity of CB and PB NK cells was related to the increased CD16 expression after incubation with IL-12 (Nguyen *et al.*, 1998). The data from our study suggests that IL-12 incubation of CBSC-NK cells does not enhance ADCC activity while a positive effect on ADCC activity from PBSC-NK cells was observed. The mechanisms responsible for this different ADCC activity between CBSC-NK cells and PBSC-NK cells might involve additional factors such as expression of adhesion molecules.

Some studies have shown that CB cells and in particular NK cells are more sensitive to IL-12 compared to BM cells or PB cells (Lee *et al.*, 1996; Condiotti and Nagler, 1998). IL-12 had a greater impact on cytokine secretion of CBSC-NK cells compared to PBSC-NK cells. This is of great importance due to the important role that IFN-γ and TNF-α play in the protection against viral infections (French and Yokoyama, 2003). However, caution should be heeded when manipulating NK cells *in vitro* to enhance NK cell functions because in many instances excessive production of IFN-γ may be detrimental by stimulating the development of autoimmune diseases (Skurkovich and Skurkovich, 2003). On the other hand, prolonged IL-12 stimulation might provide a negative feedback mechanism in NK cells (Huang *et al.*, 2011b). These mechanisms have been linked to the expression of micro-RNAs (miRNAs) (Huang *et al.*, 2011a). Additional studies in this regard will elucidate the different NK cell behaviors observed after IL-12 incubation.

In summary, the results of this chapter highlighted CD33 blocking on NK cells generated *in vitro* only affected IFN-γ secretion when CBSC-NK cells were stimulated with K562, otherwise, no effects on NK cell effector functions were observed. Conversely, our data regarding the use of IL-12 incubation suggests that NK cells from CBSC and PBSC cultures may have a different activation threshold; while CBSC-NK cells react earlier (24 h) PBSC-NK cells take up to 40 h. Importantly, IL-12 considerably enhanced cytokine secretion in CBSC-NK cells. The use of the information derived from this study may allow improved NK cell manipulation *in vitro* for immunotherapy uses.

Chapter 7: Discussion and future work

The aims of this thesis were to compare the potential of fresh and frozen CBSC to generate NK cells *in vitro* by analysing the phenotype and functionality of the generated product. Additionally, a comparison with another source of HSC was performed: PBSC. The results from this thesis show that frozen CBSC are a suitable source of HSC for NK cell generation *in vitro*, able to generate high numbers of NK cells without jeopardising cytotoxic and immunoregulatory properties. It also reports the effects of IL-12 on NK cells generated from CBSC and PBSC and the role of the myeloid marker CD33 on NK cell effector functions. This chapter discusses the data from all chapters and proposes future work and potential clinical application derived from the results obtained.

7.1 The scope of this study

One of the main challenges in assessing the feasibility of generating NK cells *in vitro* for immunotherapy is the direct comparison of the multiple variables affecting these protocols. Figure 7.1 summarises characterisations performed during this study and contributions by other groups. The majority of the current studies on NK cells generated *in vitro* focuses on the phenotypic and functional features of the generated product (figure 7.1) while a few studies focus on NK cell ontogenesis, the comparison of different HSC sources or the study of the role of specific markers on NK cell functions. This thesis focused on the areas that will likely have an impact on the design of protocols for NK cell generation *in vitro* (figure 7.1).

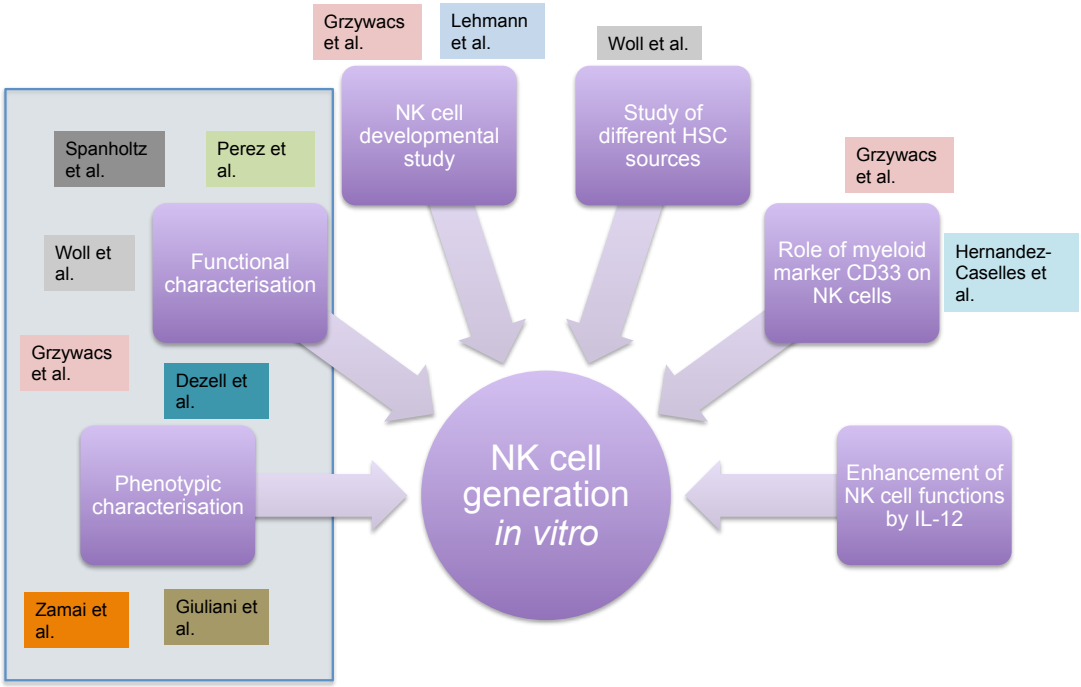


Figure 7.1. Studies of NK cells generated *in vitro*.

7.2 Natural Killer cell generation and development *in vitro* from haematopoietic stem cells

Using fresh CBSC, an initial comparison between a modified protocol and a published one (Grzywacz *et al.*, 2006) was performed demonstrating that NK cell features remained unaltered using a protocol that required less cytokines. In addition, one of the major finding of this work was the superior capacity of frozen CBSC to generate high numbers of NK cells *in vitro*. This is of major importance, as cryopreserved cells are an attractive option for clinical settings. However, different CB units were used to compare NK cell production *in vitro* from fresh and frozen CBSC. The CB units provided for this study were classified as research samples, having a lower blood volume and consequently lower HSC numbers. The access to units with high numbers of HSC or non-research samples will allow additional comparisons using CBSC from the same unit for fresh and frozen cultures and will reinforce the findings from this thesis. As NK cell therapy relies on the use of high doses of NK cells, the use of frozen CBSC will definitely suit the current demands. NK cells should be able to recognise and kill the majority of malignant cells. Currently, several groups have explored the potential application of NK cell immunotherapy in several types of cancers but NK cell immunotherapy could also be considered in the context of HSCT to enforce GvL. Clinical studies have been performed on the treatment of metastatic renal cell

carcinoma (MRCC) (Escudier *et al.*, 1994), malignant glioma (Ishikawa *et al.*, 2004), metastatic breast cancer (deMagalhaes-Silverman *et al.*, 2000), acute lymphoblastic leukaemia (ALL) (Koehl *et al.*, 2004) and CML (Passweg *et al.*, 2004). Partial results have been obtained from the aforementioned studies, as not all patients showed an improvement on survival or disease-free survival. As mentioned, the main drawbacks in these therapies are the limited numbers of available NK cells when obtained from autologous or allogeneic apheresis and the state of NK cell activation. Sufficient numbers of NK cells derived from HSC with optimal activation states could be used for these therapies.

There have been reports questioning the use of CBSC as HSC source for NK cell generation *in vitro*, as it is believed that BM or hESC are better sources for this purpose. Thus, in addition to CBSC, PBSC were included as another HSC source. Frozen CBSC have higher expansion and were able to generate higher NK cell numbers than frozen PBSC. Frozen PBSC samples were kindly provided by Dr Kwee Yong, UCL, through collaboration. When comparing the expression of NK cell receptors and NK cell effector functions between CBSC-NK cells and PBSC-NK cells high variability was observed, with a lower sample number in the latest group (n=3-6 versus n=9 from CBSC) accounting for the lack of statistical significance. Mann-Whitney test was performed for the majority of the analysis since the assumption that the sample had a normal distribution was not made. Even though other publications in the same field use student's t-test even with low number of samples (n=3) (Spanholtz *et al.*, 2011a), during this work we opted for Mann-Whitney test following statistician's recommendations. Regardless of the fact that frozen CBSC had better potential for generating NK cells compared to fresh CBSC, a similar interpretation for fresh and frozen PBSC cultures could not be made, since it was not performed. Some studies demonstrated that frozen PBSC would have impaired functions compared to fresh PBSC in the context of HSCT (Lioznov *et al.*, 2008); therefore, this study can only claim that frozen CBSC are a better source of HSC compared to frozen PBSC. Even though the use of frozen samples is ideal, the addition of experiments performed on fresh PBSC will certainly complement this work. Moreover, the comparison of CBSC to BM progenitors using the protocol in this thesis will also provide useful information to aid choice of the correct HSC source for NK cell generation *in vitro*.

During this thesis, NK cell development was followed closely using the model proposed by Freud *et al* (Freud *et al.*, 2006). In addition, the expression of TFs and markers of the lymphoid and myeloid lineages was also investigated. After integrating the data from CD56 acquisition and the expression of lymphoid precursor markers and TFs, an

interesting observation was noted: key events occurred from day 14 to day 21 in the cultures. Figure 7.2 shows the combination of these data for fresh and frozen CBSC and PBSC cultures.

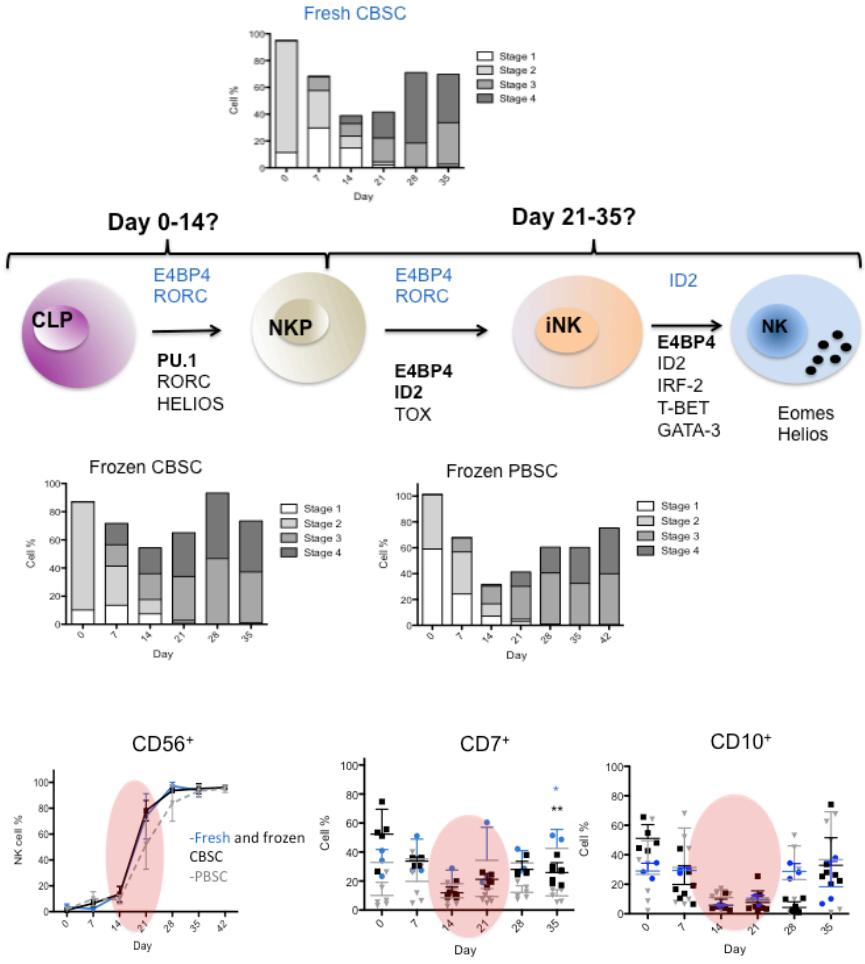


Figure 7.2. NK cell development integrating flow cytometry and molecular data. NK cell development for fresh and frozen CBSC and PBSC cultures are presented along with the expression of TFs and lymphoid lineage markers. Semi-transparent red circles highlight day 14 and day 21 in graphs.

From day 14 to 21, the expression of CD56 increased from approximately 10% to 80% and the expression of PU.1, E4PB4 and ID2 was high especially in fresh CBSC cultures. A shortcoming from the TFs mRNA analysis was the analysis of a mixture of cells (stages 1-3 and other precursors), but cell sorting could be a good strategy to overcome this limitation in the future. An interesting observation was also the lower levels of CD7 and CD10 expression at days 14 and 21. Figure 7.2 shows that NK cell stages 1, 2 and 3 were present at day 14 and only stages 2 and 3 at day 21, however only 30-60% of cells in culture comprise NK cell precursors. This suggests that other cells could have been recruited for NK cell generation. There is a possibility of recruitment of myeloid precursors to generate NK cells, as others have suggested

(Grzywacz *et al.*, 2011). Additional experiments including cell sorting and separate culture of NK cell precursors and the cell fraction that does not correspond to NK cell precursors from day 14 could reveal if indeed $CD33^{+}$ or other cells were recruited for NK cell differentiation *in vitro*.

7.3 Phenotype and functions of Natural Killer cells generated *in vitro* compared to cord blood and peripheral blood NK cells

The study of the phenotype and effector functions of NK cells from CB and PB has been performed previously, to a certain extent (Jacobs *et al.*, 2001; Dalle JH, 2005). This work included the study of NK cells subsets $CD56^{bright}$ and $CD56^{dim}$ from CB and PB NK cells (appendix 1). This publication represents a major aid hence allowing the comparison of NK cells generated *in vitro* to those present in CB and PB. It was shown during this study that $CD56^{bright}$ cells from CB have a higher expression of CXCR4, NKG2A, 2B4 and CD48 whereas $CD56^{bright}$ cells from PB showed higher expression of adhesion molecules and activating receptors. PB $CD56^{bright}$ cells had higher intracellular perforin expression and higher degranulation compared to CB $CD56^{bright}$ cells (figure 7.3). PB $CD56^{dim}$ cells exhibited higher inhibitory receptors expression, higher expression of activating receptors, L-selectin, granzyme B, perforin, IFN-γ and better degranulation compared to CB $CD56^{dim}$ cells (figure 7.3). These features are part of the trademark of cytotoxic activity in PB $CD56^{dim}$ cells. NK cell characterisation from CB, PB and NK cells generated *in vitro* are compared in figure 7.3. The data presented during this work suggests that NK cells generated *in vitro* do not precisely resemble CB or PB NK cell subsets.

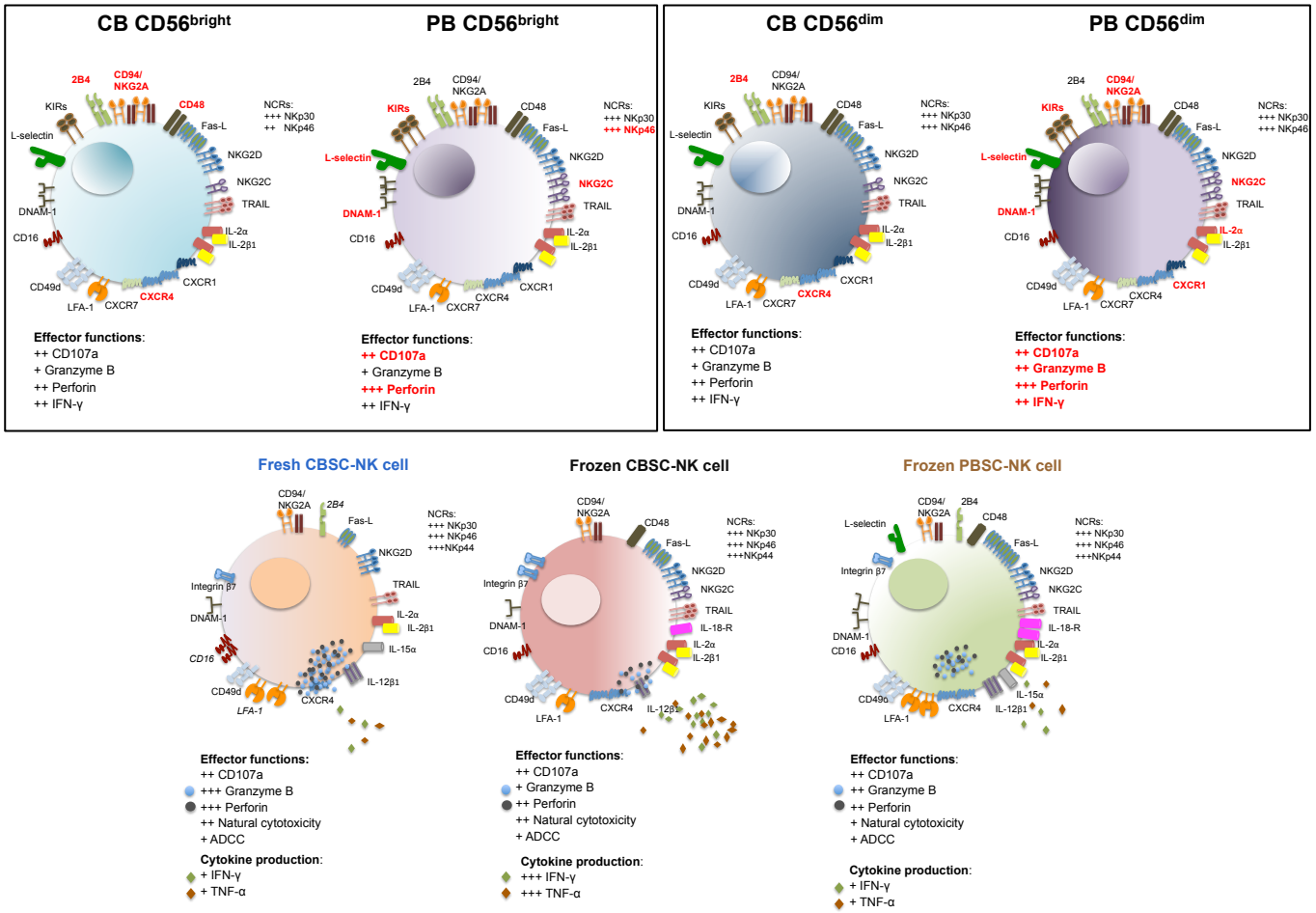


Figure 7.3. Phenotypic and functional features of CB NK cells, PB NK cells and NK cells generated *in vitro*. The upper panel shows the characterisation of CB and PB CD56^{dim} and CD56^{bright} NK cells. The receptors in bold red are more expressed compared to their counterpart (PB or CB). The lower panel shows the complete characterisation of NK cells generated *in vitro*.

Fresh CBSC-NK cells have features of PB $CD56^{dim}$ cells; these include the high expression of granzyme B, perforin and CD16 expression. However, the low expression of KIRs, CXCR1 and NKG2C suggest features characteristic of CB $CD56^{bright}$ NK cells. Similar observations in which $CD56^{dim}$ and $CD56^{bright}$ features are present were found from frozen CBSC-NK cells. For instance, an enhanced ability to kill K562 cells *in vitro* ($CD56^{dim}$ feature) and high secretion of IFN-γ and TNF-α ($CD56^{bright}$ feature). PBSC-NK cells also had $CD56^{dim}$ features in terms of granzyme B and perforin expression, but the low expression of CD16 and KIRs are $CD56^{bright}$ features.

An interesting observation during this study was the absence of KIRs expression on the generated NK cells. mRNA analysis revealed a similar transcriptional pattern from NK cells generated from HSC to CB and PB NK cells, post-translational modifications could be occurring, accounting for the differences observed by flow cytometry. These modifications include phosphorylation, acetylation, methylation and glycosylation among others. Several studies have addressed the question of KIR transcriptional regulation (Trompeter *et al.*, 2005; Cichocki *et al.*, 2011), but of special interest would be the study of post-translational modifications for KIRs mRNA. Another aspect regarding NK cell immunotherapy is the possible prediction of alloreactivity according to KIRs mismatch. Although a genotypic analysis was performed during this work (data not shown), only KIR2DL1/DS1, KIR2DL2/3 were screened in NK cells generated *in vitro* thus limiting the knowledge of potential alloreactivity. In addition, this alloreactivity should, if possible, be tested *in vitro* prior to infusion in order to select the best suitable donor HSC for generation of NK cells *in vitro*.

Additional experiments addressing the consequences of low KIRs expression in terms of education/licensing would clarify whether this could be a disadvantage. To assess NK cell education, Anfossi *et al*. tested the functions of human $KIR^{-}NKG2A^{-}$ and $KIR^{+}NKG2A^{+}$ NK cells using the same assays as used in this thesis (^{51}Cr release assay with K562, CD107a expression and IFN-γ expression) (Anfossi *et al.*, 2006). The same assays could be used in future experiments but taking into account some modifications. Since only the expression of a few KIRs were included in this thesis, further studies including the analysis of other KIRs expression (KIR2DL1, KIR2DS1, KIR2DL2, KIR2DL3, KIR2DS2, KIR2DS4 and KIR3DL1) and further sorting of KIR^{+} and KIR^{-} populations will shed light on the education/licensing status of NK cells and the therapeutic potential of $KIR^{low/-}$ cells generated in this work.

7.4 Does the phenotype of Natural Killer cells generated *in vitro* resemble that of other tissue-specific Natural Killer cells?

NK cells are not only found in PB or CB, but also in different tissues. For instance, uNK cells are characterised by their lack of expression of L-selectin, CD16 and CD57 while expressing KIRs, CD69 and being CD56 superbright. Moreover, uNK cells have been described as having low cytolytic activity against K562 cells (Ferry *et al.*, 1990). Liver $CD56^{dim}$ NK cells express KIR, NKG2A, CD69 and NKp44, and interaction with Kupffer cells can lead to cytolytic activity and cytokine production of these cells (lower compared to PB $CD56^{dim}$ NK cells) (Tu *et al.*, 2008; Burt *et al.*, 2009). NK-22 cells are characterised by the expression of NKp44 and CCR6, IL-22 secretion and do not express perforin and have little granzyme B (Cella *et al.*, 2009).

Like some of these subsets, NK cells generated *in vitro* also expressed CD69 (around 50%, data not shown), NKp44 and low levels of L-selectin. However, they were able to kill target cells *in vitro* unlike the majority of other tissue NK cell subsets that have low cytotoxic activity. A more detailed analysis reveals the lack of expression of VEGF in the generated cells as measured by qPCR (data not shown), usually expressed by uNK cells (Moffett-King, 2002; Vacca *et al.*, 2011), lack of the transcription factor GATA-3 present in thymic NK cells (Vosshenrich *et al.*, 2006) and lack of IL-22 secretion, hallmark of NK22 cells (data not shown) (Cella *et al.*, 2009). Therefore, NK cells generated *in vitro* have little resemblance with the subsets mentioned above. The microenvironment probably plays an important role in establishing NK cell phenotype. The signals that determine this process are still undefined, but it is clear that the signals giving rise to uterine, thymic, liver NK cells or NK-22 cells are probably absent in the system used in this thesis.

7.5 The myeloid marker CD33

The data presented during this thesis contributes to the notion that NK cell development might not exclusively occur from the lymphoid lineage. It was shown that $CD45^{+}CD33^{+}$ cells were present at the beginning and throughout the culture of HSC. Moreover, around 50% NK cells generated *in vitro* co-expressed CD33. The data acquired during the experiments for blocking CD33 on NK cells generated *in vitro* demonstrated that intracellular IFN-γ increased when stimulated by K562 cells. As discussed, this could be beneficial if concomitant anti-CD33 and NK cell therapy is applied. Further experiments *in vitro* could include testing of anti-CD33 commercial

drugs (Gemtuzumab ozogamizin or Lintuzumab) during the final assessment of NK cell effector functions. Other functional features tested were not affected by CD33 blocking. One of the reasons could be the internalisation of the CD33 receptor during anti-CD33 blocking. In this regard, sorted $CD33^{+}CD56^{+}$ populations ($CD33^{+}CD56^{+}$ and $CD33^{-}CD56^{+}$) could be tested for their ability to secrete cytokines, degranulate and perform cytotoxic functions against K562 and during ADCC assays. During this work it was also shown that incubation with IL-12 increased CD33 expression on NK cells generated *in vitro*. Additional experiments could be performed using PB and CB NK cells to determine if a similar effect is observed and the consequences.

7.6 Impact of IL-12

IL-12 has been shown to be a promising candidate for NK cell activation *in vitro* in our laboratory. During this thesis, the data regarding the use of IL-12 showed a different activation threshold by CBSC-NK cells and PBSC-NK cells. A complete phenotypic analysis was performed before and after incubation with IL-12 for 40 h. The results revealed that the repertoire of PBSC-NK cells was altered, while the expression of some receptors changed in CBSC-NK cells. The cytokine secretion of IFN-γ and TNF-α was improved in CBSC-NK cells with IL-12 incubation; PBSC-NK cells showed slightly increased secretion of these cytokines. Importantly, the expression of granzyme B increased with IL-12 incubation, reaching expression levels similar to those of PBSC-NK cells. The expression of perforin in CBSC-NK cells and PBSC-NK cells was not altered with IL-12 incubation. The expression of CD16 on NK cells changed with IL-12 incubation, as did ADCC activity in CBSC-NK cells and PBSC-NK cells after IL-12 incubation. The killing of K562 cells by CBSC-NK cells and PBSC-NK cells was also improved using IL-12 incubation for 24 h while a decrease in capacity was observed using 40 h in both HSC cultures. It would also be beneficial to know if cells should be activated prior-infusion with IL-12 or if IL-12 administration would have a similar effect on the generated NK cells *in vivo*. It has been reported that administration of IL-12 in the treatment of some malignancies can cause severe toxicity (Car *et al.*, 1999). Moreover, with the exception of non-Hodgkin's lymphoma (Younes *et al.*, 2004), cutaneous T cell lymphomas (Rook *et al.*, 1999) and Kaposi sarcoma related to AIDS (Little *et al.*, 2006), the efficacy of IL-12 infusion on the treatment of some haematological diseases and solid tumours has been minimal (Bajetta *et al.*, 1998; Motzer *et al.*, 1998; Wadler *et al.*, 2004). Taking into account this background the need for *in vivo* information using mouse models would help in the decision on how to administer IL-12. Non-obese diabetic severe combined immune deficient (NOD-SCID) mice are deficient in T, B and NK cells, this model allows the establishment of different

tumours and could contribute in determining whether NK cells should be activated *in vivo* or *in vitro*.

Although some of the mechanisms that regulate NK cell activation by IL-12 have been studied, this work provides further opportunities to explore additional molecular events that regulate NK cell activation during IL-12 incubation. During this thesis, RNA was extracted from NK cells generated *in vitro* incubated without IL-12 or with IL-12 for 4, 24 and 40 h (fresh CBSC-NK cells n=3, frozen CBSC-NK cells n=7, PBSC-NK cells n=6). These samples are currently stored and ready to be used for further experiments. The use of microarray technologies (Affymetrix) would shed light on the signalling molecules, transcription factors and proteins involved in NK cell activation with IL-12. Overall, IL-12 can effectively improve NK cell functions, taking into consideration that CBSC-NK cells and PBSC-NK cells hold different threshold of activation. Additionally, other cytokines either alone or in combination with others are currently under investigation in the laboratory and the findings from these studies could also be utilised to test NK cells generated *in vitro*.

7.7 Killing *in vivo* of Natural Killer cell generated *in vitro*

NK cells generated *in vitro* in this thesis exhibited immunoregulatory and cytotoxic properties. The findings of this study can be extrapolated to a certain extent to predict NK cell activity *in vivo*. However, solid evidence is limited due to the lack of functional assays using leukaemia cells *in vitro* or in an *in vivo* system. An effort to explore NK cell killing potential *in vivo* was initiated during this work using a humanised mouse model (NSG:NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ). In this model, labelled K562 cells were injected intra-peritoneally with or without injection of PB NK cells. After 48 h, peritoneal washes were performed to retrieve cells and determine NK cell killing using FACS. The labelling of K562 cells was standardised using a fluorescent cell linker (PKH67, Sigma-Aldrich) and the use of PB NK cells with or without IL-15 activation at different effector-to-target ratios was explored in three different sets of experiments. The preliminary data provide the background for further modifications of the system to successfully evaluate NK cell-mediated killing *in vivo* using this model however, because of time constraints, this work could not be done.

Access to patients' cells would also facilitate the generation of helpful information regarding the effector functions of NK cells generated *in vitro*. For instance, AML cells could be used as target cells for killing assays *in vitro*. Once the animal model is set

up, the concomitant administration of malignant cells and NK cells generated *in vitro* into mice could illuminate the cytotoxic capacities of the generated NK cells.

7.8 Parallel research using the Natural Killer cell generation *in vitro* protocol

This project was initiated with the purpose of maximising the use of CB as a source of HSC, which does not require invasive collection and for which the numbers of units available are continuously growing. The optimisation of this NK cell generation system *in vitro* was useful for other research projects and was not only limited to this study. Once the standardisation of the NK cell generation protocol was performed, additional studies were performed in parallel to this study. Figure 7.4 depicts some of these studies. The NK cell generation protocol allows the use of CBSC and PBSC for NK cell ontogeny studies. The current research in the laboratory includes the impact of immunosuppressive drugs or other cells (Tregs) on NK cell development (figure 7.4). This thesis also provides the basis for additional research on IL-12, CD33 and NK cell effector functions *in vivo*, all of which that could potentially contribute to NK cell manipulation for immunotherapy (figure 7.4).

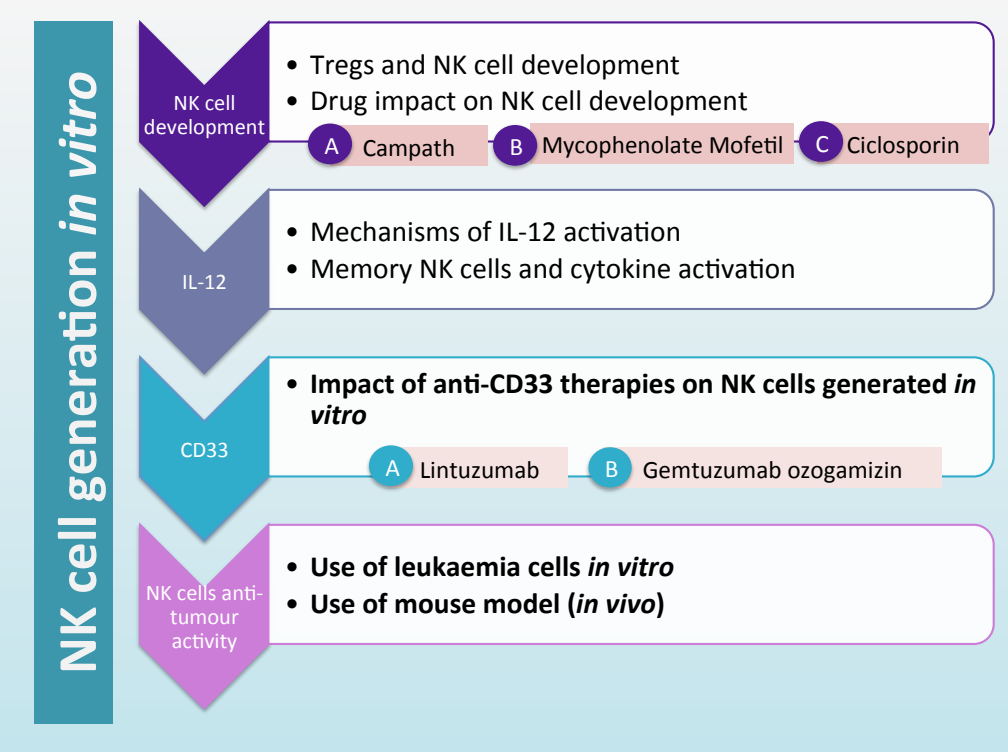


Figure 7.4. Current applications of the NK cell generation *in vitro* protocol and future directions. The figure shows the current projects performed parallel to this work and black bold sentences represent future work that will complement this thesis.

7.9 Translation to the clinic

The data presented during this work have potential clinical applications. Although these applications will certainly need additional *in vitro* and *in vivo* experiments, this thesis provides the knowledge for better decision-making regarding HSC source, cytokine mix for NK cell generation protocols and the use of cryopreserved cells that should be taken into consideration while establishing NK cell immunotherapy protocols. NK cells play a key role in the eradication of haematological malignancies, small solid tumours and even tumour metastases (Ljunggren and Malmberg, 2007; Chan *et al.*, 2008). NK cells would be more effective in scenarios in which the tumour burden is small. A correlation between NK cell doses and eradication of malignant cells has been observed previously (Clausen *et al.*, 2007; Tanaka *et al.*, 2012). Hence the importance of developing new protocols that allow the generation of high numbers of NK cells with an optimal activation state and from the appropriate HSC source.

The results from this work have a number of important implications for the design of future clinical NK cell protocols. First, it is recommended to use frozen CBSC in order to generate higher numbers of NK cells with optimal immunoregulatory and cytotoxic functions. Although the methodology used in this study involved the processing of CB units within 24 h for HSC isolation, in some instances this might not be feasible. However, previous studies have reported the optimal isolation of HSC from frozen CB units (Spanholtz *et al.*, 2011a) and similar isolations have been performed in our lab (CD133 isolation). It was also suggested to use of a cytokine cocktail with only IL-15 for the last two weeks of the culture. This modification would reduce costs in reagents and does not jeopardise NK cell effector functions. The improvement of NK cell effector functions can be achieved using IL-12 (projected to be approved by the Federal Drug Administration in 2014) and 24 h incubation prior-infusion.

Due to the introduction of new therapies involving the manipulation of HSC, several guidelines have been established by regulatory agencies in the USA and in Europe. European Medicines Agency (EMA), a decentralised agency from the European Union, is responsible for the scientific evaluation of medicines. Thus, additional considerations should be taken into account before a possible translation of this study from the "bench" to the clinic can be performed. The starting material to generate NK cells during this work was HSC, either from CB or PB. The background information of these materials should be documented, including cell banking and viral safety. This can be easily achieved as current practices in CB banks include serological tests for viral detection and HLA typing (low-resolution). HSC are a heterogeneous cell population

with variable differentiation capacity and probably are in different development stages. Thus, pre-clinical studies should include a standardised characterisation and a robust manufacturing process allowing reproducibility and consistency of the final product. Although not extensive, a characterisation including NK cell progenitors, expression of myeloid and lymphoid lineage markers and a panel for stem cell receptors was included during this study using a four-colour FACS machine. This knowledge can be easily translated into protocols for clinical use, as flow cytometry facilities are usually available in hospitals or CB banks, therefore this analysis to cryopreservation.

The next challenge is the compliance with GMP guidelines. So far, the use of murine feeder layers provides the microenvironment for optimal NK cell generation using HSC. However, these systems are a potential source of xeno-biological contamination, carrying the risk of xeno-toxicity and the presence of viral contaminations. Few studies have overcome the use of murine feeder layers and developed xeno-free systems in which NK cell generation from HSC can be obtained. These systems have been performed using CBSC, showing promising results, including the generation of high NK cell numbers with an activated phenotype able to kill K562 and leukaemia cells in *vitro*. Based on these results, a clinical trial was initiated in 2011 (NTR2818, http://www.trialregister.nl/trialreg/admin/rctview.asp?TC=2818). During these studies however, a comprehensive characterisation or the comparison of other HSC sources was not performed. EMA has highlighted a variety of murine viruses that may or may not be pathogenic in humans. While there is a lack of information regarding CBSC/PBSC susceptibility to murine viruses, Amit *et al*. have reported that hESC are resistant to certain murine viruses (Amit *et al*., 2005). Although further research would need to be performed for the feeder layer used in this work (EL08.1D2), additional alternatives arise from the fact that the murine cells die by week 3. Dead cells as well as viruses (18-100 nm) could be removed using physical methods (filtration, see appendix 4) or magnetic labeling. In order to validate and confirm the absence of murine cells, it would also be helpful to incorporate the use of commercial kits available, able to detect murine DNA (StemElitetm ID System, Promega).

NK cells should ideally be infused after reduction of tumour mass (either by chemotherapy, surgery or radiotherapy) or after immunosuppressive regimen in haematological diseases. Regarding HSC transplant (either BMSC, PBSC or CBSC), NK cell immunotherapy could be used at day 0 (transplantation day) or post-transplant. A recent phase II study has revealed that allogeneic NK cell infusions after haploidentical HSC transplantation (day +0, +44 and +100) had no significant effect on relapse or survival rates. During this study, the timing of infusions was questioned and

the importance of the purity and NK cell activation state was emphasised (Stern *et al.*, 2013). This might suggest that multiple NK cell infusions are needed in order to achieve complete remission, but this will depend on the disease treated. It should also be taken into account that *in vivo* expansion and functions of NK cells might be limited by the host, where rejection by myeloid, lymphoid and Tregs can occur (Geller *et al.*, 2011). Therefore, the secretion of molecules (cytokines, interferons, chemokines among others) by both the host receiving the therapy and the infused NK cells should be monitored and the impact on therapy outcome should be documented.

Previous studies have demonstrated the considerable alloreactivity potential that NK cells hold (Colonna *et al.*, 1993; Ruggeri *et al.*, 1999; Ruggeri *et al.*, 2002), particularly when there is KIR-ligand mismatch (donor to host). Great lessons have been learned from AML treatment, where T-cell depleted PBSC transplants from haploidentical family members were used. From the 57 patients treated, 90.2% achieved engraftment and 26% achieved event-free survival, more importantly GvHD was only present in 8.6% of the cases (Ruggeri *et al.*, 2002). Another study showed 90% event-free survival in patients with KIR ligand mismatch in HSCT from unrelated donors (Giebel *et al.*, 2003). These data provide evidence supporting the use of third party HSC for NK cell generation instead of autologous HSC. The increasing amount of CB units worldwide and the availability of this HSC source complying with viral safety and HLA typing would allow the use of these HSC for NK cell immunotherapy purposes.

A clinical trial (http://www.trialregister.nl/trialreg/admin/rctview.asp?TC=2818) started in 2011 with the purpose of determining the optimal NK cell dose for the treatment of AML in patients unable to receive HSCT. The main focus of this trial is to test the safety of NK cells infusion involving dose escalation. Although in another context, the safety of NK cells in HSCT has previously been studied. Just recently, higher doses of NK cells (>2.8 x 10^6/kg) in BMT have been reported to prevent GvHD compared to low doses (<2.8 x 10^6/kg) (Tanaka *et al.*, 2012). Moreover, even higher doses of NK cells were tested in allogeneic PBSCT ($\geq$5 x 10^7/kg), improving transplant outcomes in terms of non-relapse mortality and infectious events (Kim *et al.*, 2005a). It seems that in the context of HSCT, high doses of NK cells are safe and well tolerated. If research CB units contain 3.8 x 10^6 $CD34^+$ cells (range 1.7-8.7 x 10^6) (Spanholtz *et al.*, 2010), 2 x 10^9 NK cells (range 0.9- 4.5 x 10^9 NK cells) could potentially be generated *in vitro*. Taking into account the highest NK cell dose tested in adult patients (70 kg), around 3.5 x 10^9 NK cells would be needed. Clinical grade CB samples could provide enough starting material to supply these needs and even for multiple infusions. NK cells generated *in vitro* could be infused at different time points (day +0, +44 and +100)

(Stern *et al.*, 2013) along with or after allogenic HSCT in the treatment of haematological diseases like AML, ALL and CML. Regarding the treatment of solid tumours, 2.2 x 10^7/kg NK cells from PB apheresis have been used in the treatment of recurrent ovarian cancer and breast cancer (Geller *et al.*, 2011). Further work is needed in this field, as NK cell immunotherapy was not successful in this report and the need to augment NK cell proliferation and persistence was emphasised in this study. The use of NK cells generated *in vitro* could provide an alternative source of NK cells, although additional experiments should confirm NK cell proliferation and persistence *in vivo*.

For the purposes of generating NK cells for adoptive immunotherapy, the work presented in this thesis has demonstrated CBSC as a superior product over PBSC. In summary, the data provides evidence supporting the generation of high numbers of NK cells using CBSC that have enhanced phenotypic and functional features. For example, analysis of CBSC-NK cells revealed their potential to proliferate was due to their interleukin receptor repertoire and the high expression of activating receptors such as NKp44 and NKG2D augmented a more efficient killing capacity *in vitro* against K562 cells and higher secretion of IFN-γ and TNF-α. The success of CBSC-NK cell immunotherapy for the treatment of cancers will vary according to the expression of ligands on the malignant cells for activating NK cell receptors. Potentially, high doses of pure and activated NK cells generated *in vitro* could be used in the treatment of MRCC (Escudier *et al.*, 1994), malignant glioma (Ishikawa *et al.*, 2004), metastatic breast cancer (deMagalhaes-Silverman *et al.*, 2000), ALL (Koehl *et al.*, 2004) and CML (Passweg *et al.*, 2004). Additionally, CBSC generated NK cells could be used as immunotherapy to treat patients with chronic viral infections such as HMCV, EBV, HCV, Varicela Zoster Virus (VZV) and HSV as the cells were equipped with DNAM-1, NKp30, NKp44, NKp46 and NKG2D receptors, known to recognise ligands on virus-infected cells enabling NK cell-mediated lysis (Tomasec *et al.*, 2005; Andoniou *et al.*, 2006; Brooks *et al.*, 2006; Chalupny *et al.*, 2006). In the field of HSCT, NK cell therapy conveys promising potential benefits for the patient including less GvHD and more efficient GvL as well as more successful engraftment. Patients could receive single or multiple infusions, and with the use of CBSC, less stringent HLA-matching is required which increases availability and renders CBSC as an ideal off-the-shelf therapeutic product.

Altogether, the results from this work provide the basis for a better selection of HSC, protocol design and even enhancement of effector NK cell functions of NK cells generated *in vitro* for immunotherapy. While further research using *in vivo* models will

complement the data generated, fundamental data was retrieved regarding NK cell development and the role of the myeloid marker CD33 that will be useful for further studies in the future.

8.2 Appendix 2

CBSC

	Inhibitory receptors			NCRs				Co-stimulatory receptors		Activation receptors					Interleukin receptors					Chemokine receptors						Adhesion molecules				
Study	CD158a	CD158b	NKG2A	NKp30	NKp44	NKp46	NKp80	2B4	CD48	Fas-L	NKG2C	NKG2D	TRAIL	CD16	IL-15Rα	IL-18R	IL-2Rα (CD25)	IL-2Rβ1 (CD122)	IL-12Rβ1 (CD212)	CCR5	CCR6	CCR7	CXCR1	CXCR4	CXCR7	CD49d	DNAM-1	Integrin β7	L-selectin (CD62L)	LFA-1
Spanholtz et al. (2010)	4%	5%	50%	70%	100%	100%	0%	100%	ND	ND	0%	90%	ND	5%	ND	ND	10%	30%	%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Spanholtz et al. (2011)	ND	ND	ND	51%	60%	100%	ND	100%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Kao et al. (2007)	ND	ND	ND	ND	ND	ND	ND	ND	ND	26%	ND	ND	ND	0%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Grzywacs et al. (2006)	10%	10%	90%	87%	97%	94%	ND	ND	ND	25%	27%	96%	ND	35%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Perez et al. (2006)	2%	4%	ND	ND	ND	81%	ND	35%	ND	ND	ND	83%	ND	21%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bonnano et al. (2009)	0.1%	0.4%	ND	ND	ND	66%	ND	89%	ND	ND	ND	14%	ND	3%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Lehmann et al. (2012)	6%	4%	82%	ND	79%	ND	ND	84%	ND	ND	1%	100%	ND	23%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Woll et al. (2005)	0%	6%	58%	24%	54%	43%	ND	ND	ND	ND	ND	ND	ND	1%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Woll et al. (2009)	2%	6%	ND	ND	40%	8-%	ND	ND	ND	ND	ND	5%	ND	12%	ND	ND	ND	ND	ND	ND	ND	0%	ND	0%	ND	ND	ND	ND	1%	100%
Miller et al. (2001)	1%	21%	22%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dezell et al. (2012)	34%	34%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.40%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Beck et al. (2009)	0.40%	0.40%	15%	81%	21%	92%	ND	91%	ND	ND	1.40%	45%	ND	30%	ND	ND	ND	ND	ND	ND	ND	3%	3%	ND	ND	ND	74%	ND	65%	71%

PBSC, BM or hESC

	Inhibitory receptors			NCRs				Co-stimulatory receptors		Activation receptors					Interleukin receptors					Chemokine receptors						Adhesion molecules				
Study	CD158a	CD158b	NKG2A	NKp30	NKp44	NKp46	NKp80	2B4	CD48	Fas-L	NKG2C	NKG2D	TRAIL	CD16	IL-15Rα	IL-18R	IL-2Rα (CD25)	IL-2Rβ1 (CD122)	IL-12Rβ1 (CD212)	CCR5	CCR6	CCR7	CXCR1	CXCR4	CXCR7	CD49d	DNAM-1	Integrin β7	L-selectin (CD62L)	LFA-1
Zamai et al. (2012)	10%	10%	ND	ND	ND	ND	ND	ND	ND	100%	ND	ND	60%	18%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	100%
Woll et al. (2005)	2%	32%	79%	50%	72%	87%	ND	ND	ND	ND	ND	ND	ND	17%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Woll et al. (2009)	32%	37%	ND	ND	72%	100%	ND	ND	ND	ND	ND	20%	ND	44%	ND	ND	ND	ND	ND	ND	ND	0%	ND	0%	ND	ND	ND	ND	0%	100%
Giuliani et al. (2008)	ND	ND	ND	100%	100%	100%	ND	ND	ND	ND	ND	100%	ND	100%	ND	ND	ND	ND	ND	ND	ND	7%	ND	ND	ND	ND	ND	ND	ND	ND

ND; not determined

8.3 Appendix 3

CBSC

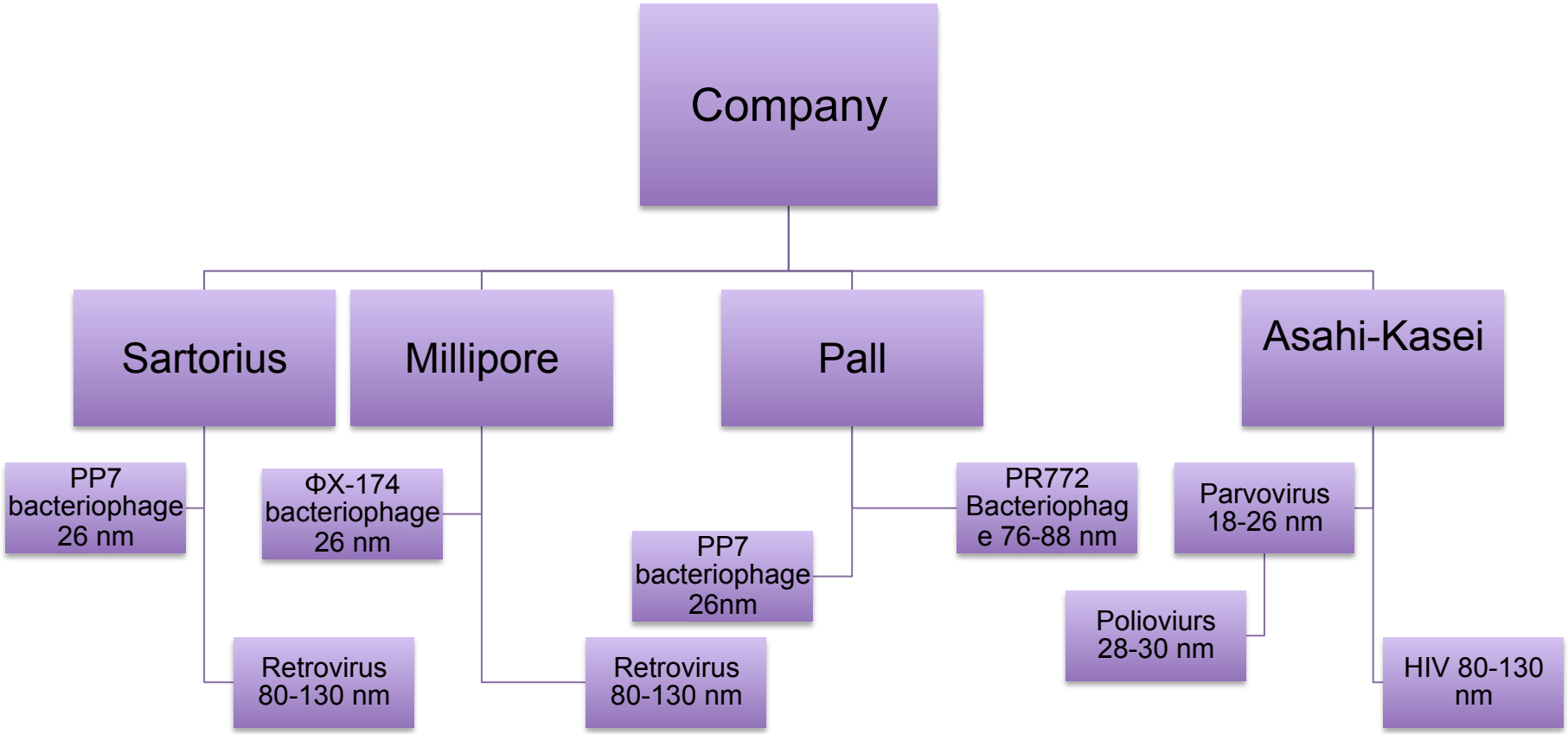
	Intracelluar				Secreted (pg/mL)				CD107a		Killing assay (various ratios)	ADCC	
Study	IFN-γ (K562)	IFN-γ (PMA&Ion)	Perforin	Granzyme B	IFN-γ (K562)	IFN-γ (PMA&Ion)	TNF-α (K562)	TNF-α (PMA&Ion)	K562 (ratio 1:1)	PMA&Ion	^{51}Cr	ADCC	Notes
Miller et al. (2001)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
Woll et al. (2005)	ND	21%	ND	ND	ND	ND	ND	ND	ND	ND	59%	ND	Killing assay ratio 10:1
Grzywacs et al. (2006)	ND	ND	78%	100%	ND	ND	ND	ND	ND	ND	40%	ND	Killing assay ratio 10:1
Perez et al. (2006)	ND	ND	54%	ND	ND	5000 (IL12+IL18)	ND	4000 (IL12+IL18)	ND	ND	60%	ND	Killing assay ratio 5:1 without IL-21
Kao et al. (2007)	ND	ND	0.1%	ND	ND	3000 (IL12+IL18)	ND	ND	ND	ND	25%	ND	Killing assay ratio 5:1
Beck et al. (2009)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	40%	ND	Killing assay ratio 15:1, delta 4.
Bonnano et al. (2009)	ND	ND	ND	ND	ND	ND	150 (no stimuli)	ND	32%	ND	ND	ND	-
Woll et al. (2009)	ND	ND	45%	60%	ND	ND	ND	ND	ND	ND	60%	ND	Killing assay ratio 10:1
Spanholtz et al. (2010)	ND	ND	ND	ND	300	ND	ND	ND	25%	ND	40%	ND	Killing assay ratio 2:1
Spanholtz et al. (2011)	ND	ND	ND	ND	ND	ND	ND	ND	20%	ND	60%	ND	Killing assay ratio 10:1, 18 h co-incubation
Dezell et al. (2012)	ND	ND	ND	ND	ND	100000 (IL-12 + IL-18)	ND	ND	ND	ND	60%	ND	Killing assay ratio 10:1
Lehmann et al. (2012)	ND	40%	ND	ND	ND	ND	ND	ND	ND	ND	60%	70%	Killing assay ratio 10:1, pre-incubation for 3 days with IL-2 (1000 U), ADCC using Europium release, ratio 12:1

PBSC, BM or hESC

	Intracelluar				Secreted (pg/mL)				CD107a		Killing assay (varios ratios)	ADCC	Notes
Study	IFN-γ (K562)	IFN-γ (PMA&Ion)	Perforin	Granzyme B	IFN-γ (K562)	IFN-γ (PMA&Ion)	TNF-α (K562)	TNF-α (PMA&Ion)	K562 (ratio 1:1)	PMA&Ion	^{51}Cr	ADCC	
Woll et al. (2005)	ND	34%	ND	ND	ND	ND	ND	ND	ND	ND	50%	ND	Killing assay ratio 10:1
Giuliani et al. (2008)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	50%	ND	Killing assay ratio 10:1, activated with IL-2
Woll et al. (2009)	ND	ND	95%	93%	ND	ND	ND	ND	ND	ND	80%	ND	Killing assay ratio 10:1
Zamai et al. (2012)	ND	ND	84%	69%	ND	ND	ND	ND	ND	ND	ND	ND	

ND; not determined

8.4 Appendix 4



8.5 Appendix 5

Publications

1. Luevano M, Daryouzeh M, Alnabhan R, Querol S, Khakoo S, Madrigal A, Saudemont A. The unique profile of cord blood natural killer cells balances incomplete maturation and effective killing function upon activation. *Hum Immunol* 2012; **73**: 248-257.

2. Luevano M, Madrigal A, Saudemont A. Generation of natural killer cells from hematopoietic stem cells in vitro for immunotherapy. *Cell Mol Immunol* 2012; **9**: 310-320.

3. Luevano M, Madrigal A, Saudemont A. Transcription factors involved in the regulation of natural killer cell development and function: an update. *Frontiers in immunology* 2012; **3**: 319.

4. Luevano M, Domogala A, Pedroza-Pacheco I, Derniame A, Escobedo-Cousin M, Querol S, Madrigal A and Saudemont A. Frozen umbilical cord blood CD34+ cells generate higher number of functional natural killer cells in vitro than mobilized peripheral blood CD34+ cells. *Blood*, manuscript in preparation for submission.

Presentations at scientific meetings

Oral presentations:

1. 2010 NIMR/UCL Seminar Day 2010. **Oral presentation**: "Generation of NK cells from cord blood stem cells, characterization and application for immunotherapy". Anatomy Building, UCL main campus, May 10th 2010.

2. Manchester, Mexican Symposium**. Oral presentation**: "Generation of NK cells from cord blood stem cells, characterization and application for immunotherapy". 2nd July 2010.

3. First CONACyT's student symposium**. Oral presentation** "Generation of NK cells from cord blood stem cells, characterization and application for immunotherapy". Paris, France. 13th-15th October 2011.

4. RCAI international program. **Oral presentation** “Generation of NK cells from cord blood stem cells, characterization and application for immunotherapy”. Yokohama, Japan. 21th-29th June 2013.

Poster presentations:

1. UCL graduate school research Poster competition 2009/2010. Poster entitled: “Generation of NK cells from cord blood stem cells, characterization and application for immunotherapy”. UCL Graduate School. March 5th 2010.

2. 3rd annual Cancer Institute Open Day/ the 1st Annual UCL Cancer Research UK Centre Conference. Poster entitled: “Generation of NK cells from cord blood stem cells, characterization and application for immunotherapy”.

3. British Immunology Society 2010. Poster entitled: “Generation of NK cells from cord blood stem cells, characterization and application for immunotherapy”. Liverpool, UK. 6-10th December 2010.

4. UCL graduate school research Poster competition 2010/2011. Poster entitled: “Generation of NK cells from cord blood stem cells, characterization and application for immunotherapy”. UCL Graduate School. 1st March 2011.

5. 2011 NIMR/UCL Seminar Day 2011. Poster: “Generation of NK cells from cord blood stem cells, characterization and application for immunotherapy”. 19th May 2011.

6. British Immunology Society 2011. Poster titled “Generation of NK cells from cord blood stem cells, characterization and application for immunotherapy”. Liverpool, UK. 5-8th December 2011.

7. Society for Natural Immunity NK2012. Poster presentation entitled “Generation of NK cells from cord blood stem cells, characterization and application for immunotherapy”. Pacific Grove, CA USA. 20th-24th April 2012.

8. NK2012 symposium. Poster presentation “Characterisation of Natural Killer cells generated from fresh and frozen umbilical cord blood stem cells”. Heidelberg Germany. 26th-29th September 2012.

9. RCAI International Summer Program. Poster presentation “Generation of NK cells from cord blood stem cells, characterization and application for immunotherapy”. Yokohama, Japan. 21th-29th June 2013

Awards

1. Award on the Primer Design sponsorship worth £3600 on qPCR reagents. Feb 2011.

2. UCL Graduate School Student Conference Fund to attend the NK2012 Society for Natural Immunity meeting. Pacific Grove, CA USA. 20th-24th April 2012.

3. Kitty Cookson Conference Travel Funds, to attend the NK2012 Society for Natural Immunity meeting. Pacific Grove, CA USA. 20th-24th April 2012

4. UCL Graduate School Student Conference Fund, to attend the NK2012 Symposium. Heidelberg Germany. 26th-29th September 2012.

5. British Society Travel Award UCL Graduate School Student Conference Fund, to attend the NK2012 Symposium. Heidelberg Germany. 26th-29th September 2012.

6. Fellowship to attend RCAI International Summer Program and summer internship, Yokohama Tokyo. June –July 2013.

7. Best presentation award of RCAI International Summer Program 2013. RIKEN centre for Integrative Medical Sciences and LGS at Chiba University. June 27 2013.

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